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Normoxic versus Hyperoxic Cardiopulmonary Bypass In Cyanotic Paediatric Patients Undergoing Cardiac Surgery
A Controlled Randomised Trial

Normoxic versus Hyperoxic Cardiopulmonary Bypass In Cyanotic Paediatric Patients Undergoing Cardiac Surgery, A Controlled Randomised Trial

By

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Abstract

During the embryonic development of the heart and the greater vessels, some malformations may occur that can result in congenital heart diseases (CHD). The incidence of CHDs is estimated to be between 8 to 10 in 1000 live births. They can be the result of an underlying genetic disorder, be related to environmental factors or a combination of both.

CHDs may require corrective cardiac surgery in order to improve life expectancy. Due to clinical advances in the recent years, the survival rate of patients with congenital heart disease has improved significantly and it is estimated that by 2020 there will be 750,000 people with congenital heart disease.

Despite advances in cardiac surgery, the current techniques in protection of cyanotic myocardium during corrective cardiac surgery are not ideal. This is particularly relevant as these hearts sustain reoxygenation injury during cardiopulmonary bypass (CPB) using higher oxygen levels. We have hypothesised that cyanotic patients' own oxygen levels during the corrective cardiac surgery could reduce myocardial and other organ injuries associated with reoxygenation insult.

In order to test our hypothesis we randomised 79 cyanotic patients to receive either *normoxic* or *hyperoxic* cardiopulmonary bypass during their corrective cardiac surgery. Systemic inflammatory responses and organ specific insults were assessed by measuring serum troponin I, alpha glutathione S-transferase (aGT), protein S100, 8-isoprostane, complement activation C3alpha, cortisol, interleukins 6, 8 and 10. The blood samples were taken at anaesthetic induction, 10 and 30 minutes after initiation of CPB, plus 10min, 4 hours and 24 hours post cessation of CPB.
We also performed a sub-analysis for cyanotic patients with double ventricular (n=47) and functional single ventricular pathology (n=32).

In all 79 patients, normoxic cardiopulmonary bypass significantly ($p<0.05$) reduced markers of organ damage (troponin I, aGT and protein S100), oxidative stress (8-isoprostane) and markers of inflammatory response (IL-6 and IL-8). In double

ventricular patients, normoxic CPB resulted in lesser levels of aGT, protein S100, 8-isoprostane, IL-10markers and cortisol. Single-ventricle patients who were randomised to receive normoxic CPB, had a significantly decreased levels of troponin I, aGT, protein S100, 8-isoprostane, C3a, IL-6, IL-8 and cortisol.

Our results provided direct evidence of the beneficial effects of normoxic versus hyperoxic CPB on the heart, brain and liver as well as inflammatory and systemic stress response in cyanotic patients undergoing corrective cardiac surgery. These findings were more prominent in cyanotic patients with functional single ventricular pathology.

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BUPA Foundation funded this project.

Dedication

I dedicate this work to *Morteza*, my uncle who left us all very unexpectedly. I will never see him again and the sorrow of his loss will always be in our hearts but our love for him will never weaken.

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award.

Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: 

Date: 15/06/2014

Publications

The Effects of Normoxic Versus Hyperoxic Cardiopulmonary Bypass On Oxidative Stress and Inflammatory Response In Cyanotic Paediatric Patients Undergoing Open Cardiac Surgery: A Randomised Controlled Trial.

Massimo Caputo, **Amir Mokhtari**, Chris A. Rogers, Nayia Panayiotou, Qiang Chen, Mohamed T. Ghorbel, Gianni D. Angelini and Andrew J. Parry

The Journal of Thoracic and Cardiovascular Surgery, 2009. 138(1): p. 206-14

Reduced Systemic inflammatory Response and Better Myocardial Protection In Single Ventricular Cyanotic Patients Undergoing Corrective Cardiac Surgery With Normoxic Cardiopulmonary Bypass.

Amir Mokhtari, Mohamed T. Ghorbel, Andrew J. Parry, Massimo Caputo

Oral presentation at the Society for Cardiothoracic Surgery in Great Britain & Ireland, Edinburgh 2014

Transcriptomic analysis of patients with tetralogy of Fallot reveals the effect of chronic hypoxia on myocardial gene expression

Mohamed T Ghorbel PhD, Myriam Cherif MSc, Emma Jenkins PhD, **Amir Mokhtari** MRCS, Gianni D Angelini FRCS, Massimo Caputo FRCS.

The Journal of Thoracic and Cardiovascular Surgery, 2010; 140:337-345

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Abbreviations:

5-HETE: 5-Hydroxyeicosatetraenoic acid

5-LOX: 5-lipoxygenase

11β-HSD2: 11β-Hydroxysteroid dehydrogenase (Type 2)

ABG: arterial blood gas

AChE: Acetylcholinesterase

ADP: Adenosine diphosphate

aGT = aGST = Alpha GST = αGT: alpha glutathione S-transferase

ALT: Alanine aminotransferase

ARDS: Acute (Adult) Respiratory Distress Syndrome

ASD: Atrial septal defect

AST: Aspartate aminotransferase

ATP: Adenosine triphosphate

AV: Atrioventricular

C3a: Complement 3 Alpha

CAT: Common arterial trunk

CHD: Congenital heart disease/defect

CK = CPK: Creatine phosphokinase

CNS: Central nervous system

COX-2: Cyclooxygenase-2

CPB: Cardiopulmonary bypass

cTnI: Cardiac troponin I

cTnT: Cardiac troponin T

CVA: Cerebrovascular accident/attack

E-C: Excitation–contraction

ECG: electrocardiography

ECMO: Extracorporeal membrane oxygenation

ELISA: Enzyme-Linked Immunosorbent Assay

eNOS: Endothelial nitric oxide synthase

FADH: Flavine adenine dinucleotide dihydrogen

FAT: Fatty acid transporter

FiO_2: Fraction of inspired oxygen

G-CSF: Granulocyte-colony stimulating factor

G6P: Glucose 6 phosphate

GLUT: Glucose transporter

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GPCR: G-protein-coupled receptors

GPx: Glutathione peroxidase

GR: Glucocorticoid receptor

(h)IL: Human interleukin

HCT: Haematocrit

HIF: Hypoxia inducible factor

HLHS: Hypoplastic left heart syndrome

hr: Hour

HRP: Horseradish peroxidase

HSP90: Heat shock protein 90

IAA: Interrupted aortic arch

Ig: immunoglobulin

IL: Interleukin

INF: Interferon

IRI: ischaemia reperfusion injury

IV: Intravenous

IVC: Inferior vena cava

kg: Kilogram

LDH: lactate dehydrogenase

MAb: Monoclonal antibody

MAPK: Mitogen activated protein kinases

mcg: Microgram

MCT: Monocarboxylic acid transporters;

MDNCF: Monocyte derived neutrophil chemotactic factor

mg: Milligram

MHC: Major histocompatibility complex

mmHg: Millimetres of mercury

MODS: Multi organ dysfunction syndrome

mPTP: Mitochondrial permeability transition pore

MR: Mineralocorticoid receptor

mRNA: Messenger ribonucleic acid

MUF: Modified Ultra Filtration

NADH: Nicotinamide adenine dinucleotide hydrogen

NADPH: Nicotinamide adenine dinucleotide phosphate-oxidase

NAP: Neutrophil activating protein

NCF: Neutrophil chemotactic factor

NCX: Sodium/calcium exchanger

ng: Nanogram

nm: Nanometre

NO: Nitrogen oxide

NOS: Nitric oxide synthase

OFR: Oxygen free radical

PDA: Patent ductus arteriosus

PDH: Pyruvate dehydrogenase;

PDK1: Phosphoinositide dependent protein kinase-1

PI3K: Phosphatidylinositol 3'-kinase

PIP2: Phosphatidylinositol (4,5)-bisphosphate

PIP3: Phosphatidylinositol (3,4,5)-triphosphate

PKB: Protein kinase B

PKC: Protein kinases C

pO_2: Partial oxygen pressure

PTA: Persistent truncus arteriosus

R&D: Research and Development

ROS: Reactive oxygen species

RPTK : Receptor protein tyrosine kinases

RyR: Ryanodine receptors

SaO_2: Oxygen saturation

SERCA: Sarcoendoplasmic reticulum calcium transport ATPase

SIR: Systemic inflammatory response

SNP: Sodium nitroprusside

SOD: Superoxide dismutase

SR: Sarcoplasmic reticulum

Streptavidin-HRP: Streptavidin, horseradish peroxidase

SVC: Superior vena cava

TAPVC: Total anomalous pulmonary venous connection

TAPVD: Total anomalous pulmonary venous drainage

TAPVR: Total anomalous pulmonary venous return

TCF: T- lymphocyte chemotactic factor

TG: Triglycerides

TGA: Transposition of the Great Arteries

TGF: Tumor growth factor

TMB: Tetramethylbenzidine

TOF: Tetralogy of Fallot

VEGF: Vascular endothelial growth factor

VSD: Ventricular septal defect

XO: Xanthine oxidase

Z-BUF: Zero-balance ultrafiltration

1 - Introduction

1.1 Background:

Patients with cyanotic congenital heart disease may have to undergo corrective cardiac surgery for the correction of their cardiac pathology. During this process these patients may undergo cardiopulmonary bypass (CPB) in order for the surgeon to be able to operate on the heart safely. The current common practice is to deliver the same level of oxygen to the cyanotic patients during CPB as it is delivered to acyanotic ones.

One of the most important goals during cardiac surgery is protection of the heart (myocardial protection). However, the techniques for myocardial protection in paediatric cyanotic hearts are not ideal [1-4]. There is a large body of evidence on the effects of re-oxygenation injury in cyanotic paediatric patients undergoing corrective cardiac surgery [5]. It has been demonstrated that cyanotic children undergoing open-heart surgery suffer myocardial damage, assessed by the release of troponin-I [5] as well as an increase in the specific markers of organ dysfunction, particularly for very susceptible organs such as the brain [3, 5-7]. Modi *et al* studied, 29 children with or without hypoxic stress. They reported that cyanotic patients exhibited significantly higher levels of troponin-I release compared to acyanotic ones [5]. They proposed that this could be due to the initial introduction of relatively high levels of oxygen during CPB. This has been also suggested in other clinical studies [2, 4, 8].

Myocardial dysfunction, characterised by endothelial damage, reduces cardiac compliance, which subsequently leads to low output syndrome. This remains a leading cause of perioperative morbidity and mortality after successful repair of congenital heart defects [1, 4, 8, 9].

Cerebral dysfunction is also another major cause of mortality and morbidity after repair of congenital cardiac defects in infants and children [6, 7]. Subtle neurological and cognitive dysfunction may be evident even among children in whom cardiac repair is completely successful. Transient neurological

derangements immediately after CPB may occur in as many as 64% of patients when studied within 24 hours of surgery [6, 7].

Little is known on the effects of controlled re-oxygenation during CPB in children undergoing corrections of congenital cardiac abnormalities.

Studies of hypoxaemia-reoxygenation in immature piglets have provided evidence for oxygen-mediated myocardial injury as a result of hyperoxaemia in the setting of previous cyanosis [10]. The immature heart has a higher tolerance to hypoxia, but preceding hypoxia prior to ischaemic cardioplegic arrest results in poor functional recovery and is associated with derangement in several metabolites [11, 12]. Hypoxia also reduces the antioxidant reserve capacity, leading to a greater susceptibility to the oxidative stress of ischaemia (e.g. cross-clamping of the aorta) and re-oxygenation [1, 4, 9, 11, 12]. Re-introduction of oxygen exacerbates this situation and eventually leads to a reduction in myocardial contractility. This is via the production of free radicals and subsequent lipid peroxidation, cell damage, mitochondrial dysfunction and enzyme leak [1, 4, 9, 13-16].

One of the strategies proposed to avoid this situation is the use of controlled re-oxygenation. This involves starting CPB with a hypoxic prime, until normoxic reoxygenation is started. This has been shown to avoid reoxygenation injury and led to almost complete functional recovery [17, 18]. Ihnken *et al* showed the beneficial effects of normoxic reoxygenation in a clinical animal model of acute hypoxaemia [19]. The same authors demonstrated that hyperoxic CPB during cardiac operations in adults resulted in oxidative myocardial damage related to oxygen-derived free radicals and nitric oxide [20]. Reduced oxygen tension management can markedly limit these adverse effects.

The concept of normoxic CPB can therefore be applied to surgical advantage during cardiac operations, especially in paediatric patients undergoing repair of complex cyanotic heart defects. Nevertheless, there is no clinical evidence showing the benefits of these surgical strategies during paediatric heart surgery.

1.2 Overview:

The heart is a vitally life-sustaining myogenic organ found in all animals with a circulatory system (including all vertebrates), and with repeated rhythmic contractions it pumps blood throughout the blood vessels. In an adult human, on average, the heart weighs 250 to 300 grams in females and 300 to 350 grams in males and it is roughly about the size of the individual's fist. It is estimated that in an average adult, the heart pumps more than 6000 litres of blood daily through the body and it beats more than three billion times over the course of their life [21-23].

The heart is principally composed of *cardiac muscle* (involuntary striated muscle tissue found in this organ only) plus *connective tissue*. Its blood supply is from coronary circulation and a double-walled sac called the pericardium encloses it. This sac shields the heart, anchors its surrounding structures, and prevents it from overfilling with blood [23, 24].

Mammalian heart has a complete division into two pumps known as *right* and *left heart* [25]. The function of the right heart is to collect less oxygenated blood from the body (via inferior and superior vena cavae as well as the coronary sinus) and send it into the lungs (*pulmonary circulation*) so that carbon dioxide can be exchanged for oxygen (*gas exchange*). The left heart receives oxygen rich blood from the lungs and pumps it out to the rest of the body (*systemic circulation*) (Figure 2).

Sometimes malformations during embryonic development can lead to *congenital heart diseases (CHD)*. Congenital heart diseases are described as abnormalities of the cardiocirculatory structure or function that exist at birth, even if it is discovered much later [26, 27]. Congenital cardiovascular malformations usually result from altered embryonic development of a normal structure or failure of such structure to progress beyond the early stage of embryonic or foetal development [27].

The true incidence of congenital cardiovascular malformations is difficult to accurately determine, however it is estimated that between 8 to 10 in 1000 live births are complicated by a cardiovascular malformation [26, 27]. Nevertheless, it is important to bear in mind that these figures do not take into account what may be the two most common congenital cardiac anomalies; the congenital, functionally normal bicuspid aortic valve and prolapse of the mitral valve [26].
CHDs can occur with Mendelian inheritance directly as a result of a genetic abnormality, be strongly associated with an underlying genetic disorder (e.g., trisomy), be related directly to the effect of an environmental toxin (e.g., alcohol), be associated with infections (e.g. rubella), or result from an interaction between multifactorial genetic and environmental influences [27].

Congenital heart diseases can be categorised into cyanotic and acyanotic. Cyanotic patients have a lower than normal arterial oxygen saturation and may present with blue appearance of the skin and mucous membranes, however acyanotic patients, have normal arterial oxygen saturation. In principal, malformations causing a left to right shunt are acyanotic and malformations causing right to left shunt are cyanotic CHDs.

Despite advances in cardiac surgery, the current techniques in protection of cyanotic myocardium during corrective cardiac surgery are not perfect [8, 28]. This is particularly relevant as these hearts sustain reoxygenation injury during cardiopulmonary bypass (using normal oxygen saturation) and prior to cardioplegic arrest [5]. We have hypothesised that cyanotic patients' own oxygen levels during the corrective cardiac surgery could reduce myocardial and other organ injuries associated with reoxygenation insult and optimise the outcome of surgery.

It is important to have an understanding of cardiac anatomy, development and physiology to appreciate the cyanotic heart. This chapter presents a brief description of cardiac anatomy, embryology and some cyanotic cardiac anomalies as well as the physiology of the heart. At the end of this chapter, the principle of cardiopulmonary bypass will be dicussed.

1.3 Anatomy of the heart and it's development

The heart is situated in the thoracic cavity in the middle mediastinum. It is a muscular organ that is hollow and somewhat pyramidal in shape. It is ensheathed in a sac called *pericardium*. Pericardium encloses the heart and the roots of the great vessels. At its base, the heart is connected to the great blood vessels and otherwise sits free within the pericardium. It has three surfaces: anterior, posterior and inferior. A normal heart has four chambers: two *atria* and two *ventricles* plus four valves. These valves work as a one-way passage for blood to flow from the atria to the ventricles and from the ventricles to the main vessels exiting the heart, called the *aorta* and the *pulmonary trunk* (Figure 1).

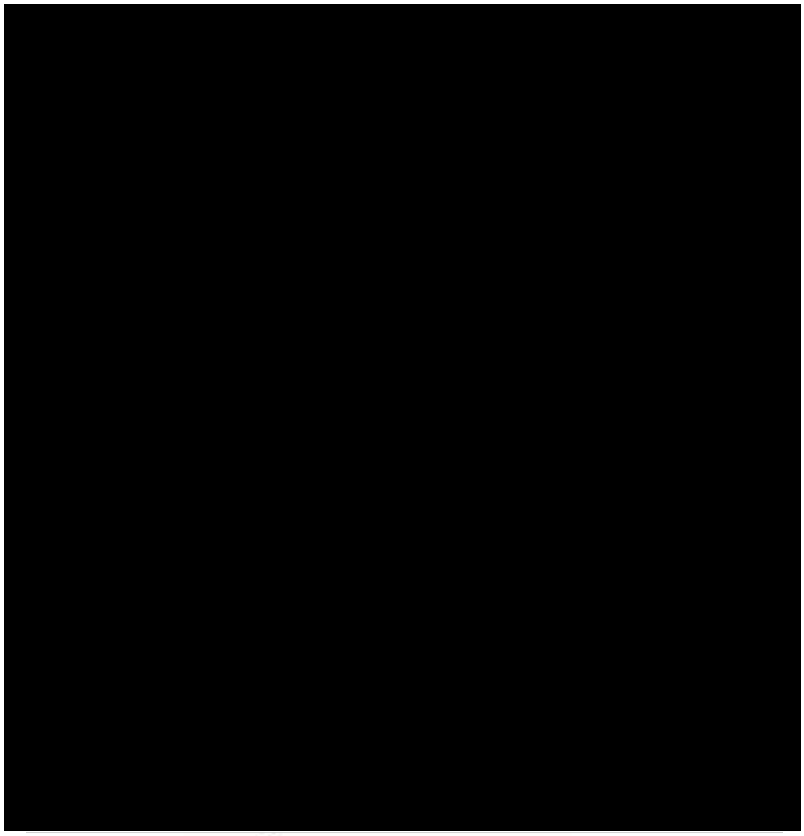

Figure 1 Chambers of the heart
(Image from www.umm.edu, University of Maryland Medical Centre)

Although the cardiac atria and ventricles are positioned posteriorly and anteriorly, due to their primary embryological positions they are referred to as right and left atria and ventricles [23]. The right atrium and right ventricle (also known as the *right heart*) form part of the heart that receives the venous

drainage that has a lower level of oxygenated blood and pumps it to the *pulmonary circulation*. During this process blood is oxygenated in the lungs. The left atrium and left ventricle (also known as the *left heart*) receive the oxygenated blood from the lungs and pump it into the *systemic circulation* to provide oxygen and nutrients to the body (Figure 2).

Figure 2 Blood Circulation
(Image inspired from Clinical Anatomy by Snell, 7th Edition[23])

The process that heart fills with blood and empties is referred to as the *cardiac cycle*[23]. In a normal adult, the heart beats between 60 to 100 times per minute at rest and in the new-born between 110 to 150 [29]. This rate can increase during exercise or some other conditions that put the body under stress such as sepsis [23].

1.4 Embryological development of the heart

During the initial 20 days of development, the human embryo lacks cardiovascular structure. Over the following 4 weeks, the development is almost completed and the appearance is very similar to that at full gestation. During this process isolated angiogenic cell islets are transformed into a complex four-chambered structure and by the 28th day blood circulation through the embryo commences [30].

Embryologically, the heart derives from the lateral mesodermal layer [30]. Initially clusters of cells arise in the mesenchyme. These clusters of cells form a plexus of endothelial blood vessels that fuse to form the right and left *endocardial heart tubes*. The first indication of any cardiovascular development occurs on approximately day 18 or 19. The right and left endocardial heart tubes soon fuse to form a single median endocardial tube. The heart tube then starts to bulge into the pericardial cavity and the *primitive heart* is established. It has been reported that when heart is cultured in vitro, the heart will loop even when the pericardial sac is removed [31]. The process of looping is therefore thought to be a genetic property of the myocardium and not related to differential growth.

The heart begins to beat soon after the 3rd week of gestation but the circulation does not start until the 4th week [23]. The heart tube then undergoes differential expansion so that several dilatations result: the *bulbus cordis* (the distal part of bulbus cordis is known as *truncus arteriosus*), the *ventricle*, the *atrium* plus the *right and left horns of sinus venosus* (Figure 3). Outside the caudal end of the pericardial sac, lay the primitive atrium and sinus venosus and outside the cranial end of the pericardial sac, lay the truncus arteriosus. Some publications however support the use of *inlet*, *outlet*, and *arterial segments* as proposed by Anderson and Becker [32-34].

Figure 3 Heart tube differentiation
(Image from www.ans.iastate.edu, Iowa State University)

The tube then begins to bend in an S shape manner with the atrium lying posterior to the ventricle (Figure 4). Tumour growth factor β (TGF β) and vascular endothelial growth factor (VEGF) signalling have been implicated in this event [35, 36].

Figure 4 Heart tube bends in an S shape form, placing atria posteriorly and ventricles anteriorly
(Image from www.ans.iastate.edu, Iowa State University)

Sinus venosus develops into the veins and some parts of the atria and the truncus arteriosus develops into the aorta, pulmonary trunk and some parts of

the ventricles. In the middle of the sinus venosus and truncus arteriosus, the heart tube develops into the remaining part of the heart [23].

During the fifth to eighth week of development, the atrioventricular valves are formed [37]. Initially, at the atrioventricular junction, the endocardial cushion tissue forms bulges. These bulges have the appearance of valves, and although they may play a significant part in the final construction of the atrioventricular valves, endocardial cushion tissues are not the precursors of the mitral and tricuspid valves [38]. A fraction of epicardial cells invades the underlying myocardium which give rise to atrioventricular valve tissue, cardiac fibroblasts and endothelial plus smooth muscle cells of the coronary vessels [39]. Coronary vascular network is formed from the epicardium [40].

As the heart continues to develop, atrial septation starts when the common atrium becomes indented externally by the bulbus cordis and truncus arteriosus. Internally, this indentation corresponds with a thin sickle-shaped membrane developing in the common atrium on day 35 [34]. This membrane later divides the common atrium into left and right atria. This membrane is known as the *septum primum*. The space between the septum primum and the endocardial cushion is known as the *foramen primum*. It is important to appreciate that the foramen primum is not a hole in the septum primum but rather a space or a gap between the septum primum and the endocardial cushion.
As the septum primum continues to grow towards the endocardial cushion, during a programed cell death (apoptosis) an opening is made in the septum primum. This opening is known as the *foramen secondum* [23] (Figure 5).

Figure 5 Atrial septation
(Image from www.alfaisaldoctors.com)

These foramens allow blood to travel from the right side of the heart to the left. This is vital for the infant since they do not breathe and all the oxygenated blood from the mother comes to the right atrium (**Error! Reference source not found.**). Meanwhile, another sickle-shaped membrane known as septum secondum develops on the anterosuperior wall of the right atrium, just right to the septum primum and left of the sinus venosus valve. Its development halts when it has just gone past the foramen secondum. The septum secondum is thicker and more rigid than the septum primum. The pressure in the right atrium forces the septum primum to be pushed away from the septum secondum. This creates an opening known as foramen ovale (named after its oval shape).

In the meantime a muscular ridge grows from the apex of the embryonic ventricle towards the aorticopulmnary septum. This muscular ridge is the embryonic ventricular septum. The aorticopulmonary septum, also known as the spiral septum, is a septum that divides the truncus arteriousus longitudinally. It is referred to as spiral septum because during its development it twists around its axis, which will lead to placing the aorta on the left and the pulmonary trunk on the right (Figure 6).

Figure 6 aorticopulmonary septum dividing truncus arteriousus into aorta and pulmonary trunk whilst twisting 180 degrees around its axis
(Image from Clinical Anatomy by Snell, 7th edition [23])

The part of the ventricular septum that is made from the embryonic ventricular septum is referred to as the *muscular inter-ventricular septum* and the part that is made from the aorticopulmonary septum is known as *membranous intra-ventricular septum* [30].

After birth once the baby starts to breathe, the resistance in the pulmonary artery drops as well as the right atrial pressure. This results in a higher pressure in the left atrium than the right. This higher pressure pushes the septum primum towards the septum secundum, which leads to the closure of the foramen ovale.

At times, during the embryonic development of the cardiovascular system, variations can occur that may lead to congenital heart defects.

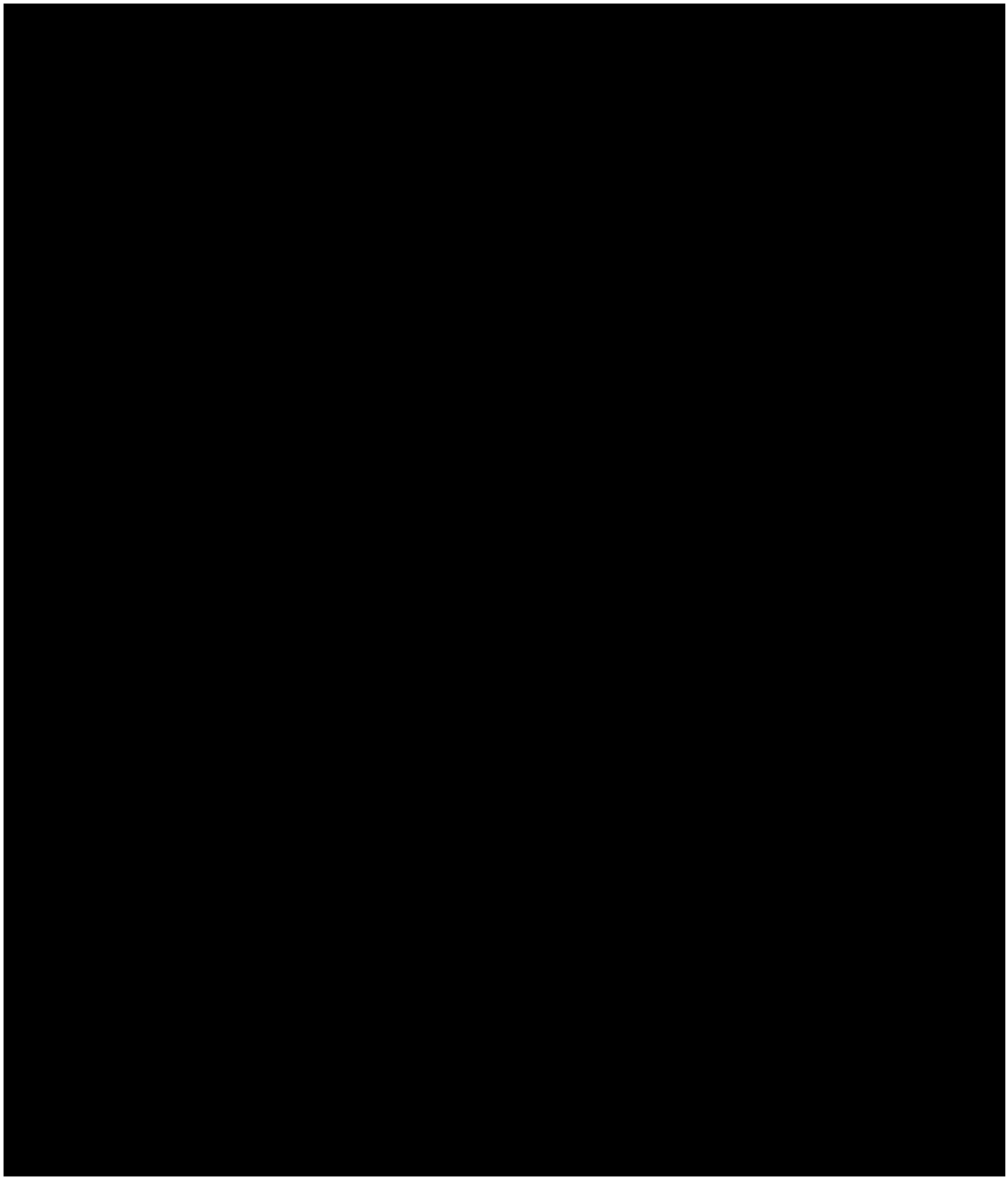

Figure 7 Foetal circulation

(Image from www.cardiachealth.org)

1.5 Congenital Heart Defect (Disease)

During the embryonic development of the heart and the greater vessels, some malformations may take place that may result in congenital heart diseases (CHD). Most such disorders arise from faulty embryogenesis during gestational week 3 thorough 8, when the major cardiovascular structures develop [41]. Although all causes of CHD are not entirely known to us, factors such as environment (for example, congenital rubella infection) and genetics (familial forms of CHD, chromosomal abnormalities such as certain mutations, trisomies 13,15,18 and 21 plus Turner syndrome) are known to have an association with CHD [41].

The incidence of CHD has been reported to be approximately between 0.8 to 1% of live births. This incidence however, can be higher in premature infants [42]. Congenital heart diseases can be categorised into cyanotic and acyanotic [41, 43, 44]. Fundamentally, malformations causing a left-to-right shunt are acyanotic and malformations causing right-to-left shunt are cyanotic CHD. The most common congenital cardiac anomaly at birth is ventricular septal defect (VSD) followed by atrial septal defect (ASD) [41]. However VSD and ASD are both acyanotic conditions as the shunt is left-to-right. Cardiac malformation associated with right-to-left shunts, are distinguished by cyanosis at, or near, the time of birth [41, 43, 44].

Due to clinical advances in the recent years, the survival rate and life expectancy of patients with congenital heart disease has improved significantly [45]. In fact, it is estimated that by 2020 there will be 750,000 people with congenital heart disease [41].

1.5.1 Cyanotic Congenital Heart Defects (Disease)

Cyanotic conditions or *blue baby syndrome* can be caused by a *right-to-left* shunt or malposition of the great arteries [44]. Cyanotic drives from the Latin word *cyano,* which means blue. The skin or mucous membranes of these patients may appear purple or blue (cyanotic), due to deoxygenated blood bypassing the lungs and entering the systemic circulation.
Some of the congenital cardiac conditions that can cause cyanosis are described below:

1.5.1.1 Tetralogy of Fallot:

Tetralogy of Fallot is the most common cyanotic cardiac defect and accounts for about 5% of all congenital cardiac malformation [46] with an incidence of 3.26 per 10,000 live births, per year [47-49]. In this malformation the division of the truncus arteriosus does not take place at the middle which then leads to: infundibular pulmonary stenosis, a conoventricular septal defect, dextroposition of the aorta so that the aortic root overrides the crest of ventricular septum, and subsequently right ventricular hypertrophy (Figure 8). This combination of anomalies was described in detail by Fallot in 1888 [50]. In addition to these four defining features, there may be pulmonary valve atresia. This is known as *tetralogy of Fallot with pulmonary atresia*, previously called truncus arteriosus type IV or pseudotruncus. It represents approximately 20% of all tetralogy of Fallot patients.

Patients with Tetralogy of Fallot are cyanotic due to right-to-left shunting at the ventricular level. This occurs because the anatomic right ventricular outflow tract obstruction causes a rise in pressure that exceeds the systemic vascular resistance. Under these circumstances, the oxygen-poor blood in the right ventricle shunts across the defect into the left ventricle and from there into the systemic arterial circulation.

Mutations in several human genes have been identified in Tetralogy of Fallot, *NKX2.5*[51], *JAG1*[52, 53], *TBX5*[54], *FOXC2*[55]. Deletion of human *TBX1* appears to be the basis for the 15% of Tetralogy of Fallot attributable to chromosome 22q11.2 micro-deletion, although *TBX1* mutations in non-deleted tetralogy of Fallot patients remain to be identified [56-58].

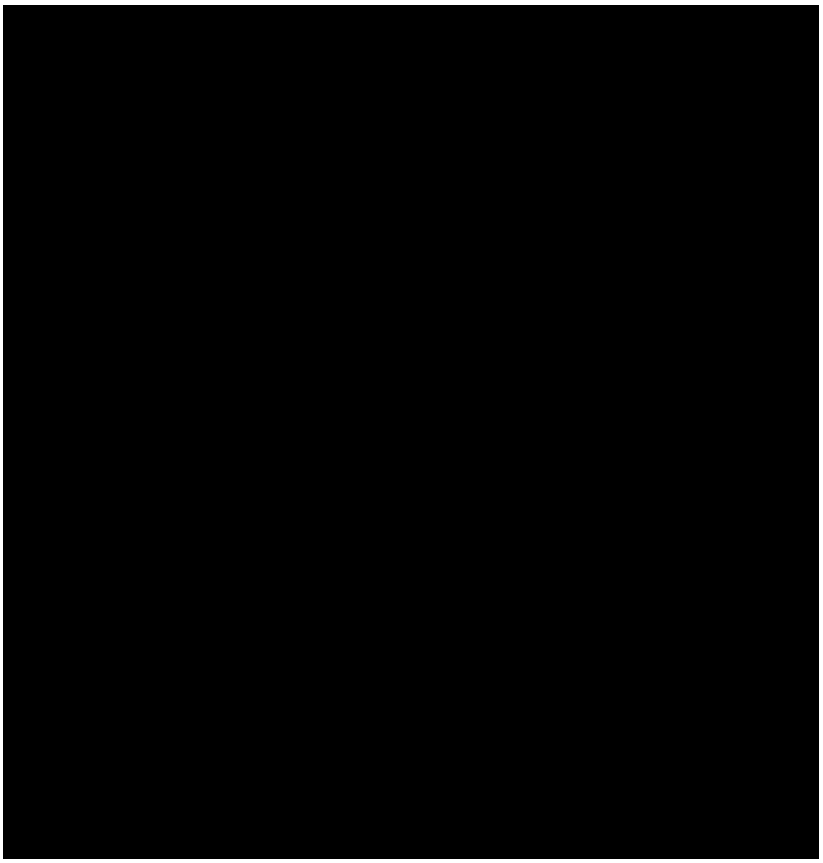

Figure 8 Tetralogy of Fallot
(Image from www.theheart.org)

1.5.1.2 Transposition of the Great Arteries (vessels) or TGA:

The second most common cyanotic congenital cardiac defect encountered in early days after birth is transposition of the great arteries. It is one of the major reasons for admission to a cardiac unit in the first two weeks of life [59]. In this condition, the natural rotation of the truncus arteriosus around its axis does not take place. This then leads to the aorta exiting from the right ventricle and the pulmonary trunk from the left ventricle (Figure 9). In utero this may not cause problems, however, this is incompatible with life after birth. Therefore for the

new-born with TGA to be able to survive, she/he has to have either a patent ductus arteriosus, a patent foramen ovale, a VSD or a combination [60].

Remarkably, the number of children born each year with TGA (~0.24/1000 live births) is constant compared to other congenital cardiac defects. This constancy may be due to an easy diagnosis in early infancy [59]. There is no data to link an identified genetic abnormality to explain the existence of TGA. Recurrence in the same family is virtually unrecognised. There are fewer extracardiac anomalies associated with TGA when compared to most other congenital cardiac defects, in fact, possibly fewer than the general population [61]. Furthermore, there is a smaller incidence of TGA among premature infants and those with low birth weights [61, 62]. However there are reports that advanced maternal age and higher birth order can be associated with higher incidence of TGA [62].

50% of the infants with TGA present with ventricular defects at birth, but in the first one year this number is reduced to one third due to spontaneous closure [63]. Additional cardiac anomalies are more common amongst children with transposition of the great arteries and ventricular septal defect compared to ones with transposition of the great arteries and intact ventricular septum. Thus, in the presence of ventricular septal defect, it is more likely to encounter pulmonary stenosis, pulmonary atresia, an overriding or straddling atrioventricular (AV) valve, coarctation of the aorta, and interruption of the aorta. However right ventricular outflow obstruction is uncommon [64].

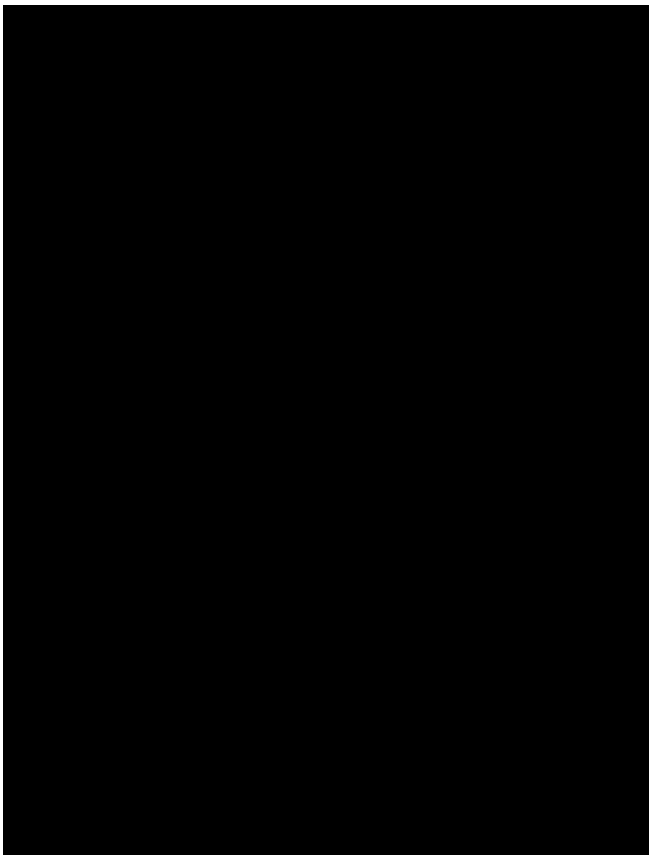

Figure 9 Transposition of Great Arteries

(Image from www.childrenshospital.org, Boston Children's Hospital)

1.5.1.3 Total Anomalous Pulmonary Venous Connection (TAPVC):

Also known as *total anomalous pulmonary venous drainage (TAPVD)* or *total anomalous pulmonary venous return (TAPVR)*, is another form of cyanotic CHD in which there is a malposition of all four pulmonary veins that leads to their abnormal connections to the systemic venous circulation.

Normally by the end of the first month of the embryological phase, lung buds have developed within the splanchnic plexus. The splanchnic plexus has numerous connections with the cardinal and omphalovitelline systems. Shortly after, a common pulmonary vein appears that connects the sinoatrial portion of the heart to the pulmonary venous plexus, and later incorporates into the left atrial wall, by which time the pulmonary–splanchnic venous connections have disappeared [65]. When the common pulmonary vein fails to unite with the left atrium, there may be some persistency in pulmonary–splanchnic venous connections. This allows pulmonary venous return at various systemic venous–right atrial levels, such as the left innominate, portal, and coronary sinus levels.

Various combinations of pulmonary vein(s) draining anomalously into the systemic venous or right heart circulation may result in left-to-right shunt. The patient is said to have TAPVC, if all pulmonary veins drain into the systemic venous circulation. There may be variable, absolute, or relative obstruction of the pulmonary venous system [66, 67]. In the presence of obstruction, there is pulmonary venous hypertension and reactive pulmonary arterial hypertension, which can often be above the systemic pressure.

It is reported that amongst critically ill infants, isolated TAPVC is the 12th most common cardiac defect (0.056 per 1000 live births), constituting 2.6% of the total prevalence [47, 61, 68]. Other simple cardiac anomalies, such as ventricular septal defect, may rarely be associated with total anomalous veins; however, major cardiac abnormalities are more likely to be present when there is asplenia and polysplenia

There are four TAPVC variants:

- *Supracardiac*: All pulmonary veins drain to the common pulmonary vein behind the left atrium, which then drains upward on the left side of the chest, usually in front of the pulmonary artery, into the left brachiocephalic (innominate) vein. Occasionally the common pulmonary venous channel drains directly into the superior vena cava and sometimes into the azygous system.

- *Cardiac*: All pulmonary veins drain into the common pulmonary vein and subsequently into the right atrium or, more often, into the coronary sinus.

- *Infradiaphragmatic*: Once pulmonary veins drain into the common pulmonary vein behind the heart, it passes down a venous channel through the diaphragm to the portal vein, ductus venosus, or hepatic vein, re-entering the heart through the left inferior vena cava.

- *Mixed:* Is a mixture of the above. For pulmonary veins to drain into the venous circulation, any anatomical combination is possible. For example, right atrium may receive the right-sided veins and the left veins to drain into the left innominate.

Following information gathering from large surgical series, the pulmonary venous return sites were reported as; 49% supracardiac, 25% cardiac, 18% infradiaphragmatic, and 8% mixed [69-73].

1.5.1.4 Hypoplastic Left Heart Syndrome (HLHS):

In this form of cyanotic CHD there is a diminutive left ventricle with underdeveloped mitral and aortic valves [74]. The prevalence varies between 0.21 and 0.28 per 1000 live births [42, 59].

Although some babies may present with severe aortic stenosis, usually there is hypoplasia or atresia of the aortic valve. Similarly, the mitral valve may be hypoplastic, severely stenotic or atretic (Figure 10). As the left ventricle is too small, it is unable to support the systemic circulation, therefore both pulmonary and systemic circulation will rely on the right heart. For a period, the right ventricle may be able to cope to support the circulation to both the lungs and the body, but if left untreated, this extra workload will eventually lead to heart failure.

As in most forms of congenital heart disease, the intrauterine circulation is adequate to meet the needs of the developing foetus in the hypoplastic left heart syndrome and to support normal intrauterine growth. However a patent ductus arteriosus (PDA) with or without an ASD must be present for the newborn to be able to survive after birth.

Aortic coarctation is a common finding amongst survivors following surgical intervention, however autopsies of untreated infants rarely show coarctation of the aorta. This suggests that there is an intrinsic tendency to develop coarctation in this anomaly [75, 76]. Hypoplastic left heart syndrome may also be associated with some cerebral anomalies [77].

Figure 10 Hypoplastic left heart
(Image from www. mediocritycodex.blogspot.co.uk)

1.5.1.5 Persistent Truncus Arteriosus (PTA):

Also known as Common Arterial Trunk (CAT), PTA is characterised by origination of a solitary arterial vessel from the heart, overriding the ventricular septum, and supplying the systemic, pulmonary and coronary circulations from the proximal ascending vessel. Reported incidence of PTA rates from 0.006 to 0.043/1000 live births [78, 79] and there is a well-recognised association with DiGeorge syndrome and deletion of chromosome 22q11 [80].

In the normal embryo, by the end of the fifth week, septation of single truncus arteriosus occurs, resulting in aorta and main pulmonary artery as well as formation of aortic and pulmonary valves. Soon after, the formation of conal septum is complete. Through these weeks, developmental disturbances can result in conotruncal abnormalities including truncus arteriosus [81-83].

For one to confidently diagnose the true truncus arteriosus, no remnant of a separate main pulmonary artery connected to the heart should be present and there must be no evidence of a separate pulmonary valve.

It is very rare for the truncal valve to be found normal and it often has deformed and thickened leaflets (72%) [84] which are variably stenotic or, more often, incompetent (50%) [85]. The number of leaflets can vary. In a literature review by Fuglestad *et al*, 21% quadricuspid, 69% tricuspid, 9% bicuspid, and 1% unicuspid were reported [85]. In almost all patients with PTA, ventricular septal defect is present, which is normally not small. Other conditions such as interrupted aortic arch (11-14%) [84] and extracardiac anomalies (21-48%) [61, 84] may be present.

1.5.1.6 Tricuspid Atresia:

The characteristics of this cyanotic CHD are the absence of the tricuspid valve plus right ventricular hypoplasia. Tainer *et al*. have reported a frequency of 0.057 per 1000 live births [61].

As there is a lack of an A-V connection, in order to maintain blood flow, an ASD as well as a VSD must be present since the right ventricle is hypoplastic. A PDA is usually present to increase pulmonary flow.

Conventionally, patients with tricuspid atresia are divided into 3 groups:

Type I: those without transposition of the great arteries,
Type II: those with transposition of the great arteries,
Type III: those with other complex anomalies.

In these patients, blood passes from the right atrium to the left atrium through an atrial septal defect or, more often, a patent foramen ovale and from there to the left ventricle. In most cases there is no indication that a valve was ever present or that there was an alignment of atrium towards the right ventricle [86].
These patients all have small right ventricles and in some cases, the right ventricle is no more than a channel connecting the left ventricle to the pulmonary artery.
A common finding is aortic coarctation, particularly in type II (about 33%). Some other cardiac anomalies also occur amongst both types I and II such as; right aortic arch, left juxtaposition of the atrial appendages, persistent left superior vena cava and right aortic arch [87, 88].

1.5.1.7 Interrupted Aortic Arch (IAA):

This is a very rare form of CHD affecting 3 per million live births [89]. The characteristic of this anomaly is the loss of luminal continuity between the descending and ascending segment of the aorta. By enlarge, IAA is associated with intracardiac malformations such as VSD, PDA, bicuspid aortic valve, left ventricular outflow tract obstruction, or aortopulmonary window. There are 3 types of IAA. Type A, where aortic arch discontinuity is distal to the left subclavian artery, type B, where the discontinuity is between left subclavian and the left carotid arteries and type C, where the discontinuity lies between left carotid and the innominate arteries. The most common type is B (53%), followed by A (43%) and C (4%) [90]. This condition is also often associated with DiGeorge Syndrome.

1.5.1.8 Pulmonary Atresia:

This form of CHD is characterised by complete obstruction of right ventricular outflow plus a variable hypoplasia of the right ventricle and tricuspid valve. The pulmonary valve cusps are obstructed with a layer of tissue resulting in no blood flow to the lungs via the pulmonary trunk. This condition is not life threatening during the intrauterine period, however following birth the baby will be cyanotic. Pulmonary atresia can exist in the presence of a VSD or an intact ventricular septum. In the latter a PDA would be the only source of pulmonary blood flow.

The incidence rate has been reported as 0.040 and 0.045 per 1000 live births, with some resent decline possibly due to termination of pregnancy [91, 92].

There are strong suggestions that pulmonary atresia in the presence of intact ventricular septum is an acquired disease rather than abnormalities related to embryological development. Supporting evidences for this theory include; large

pulmonary arteries despite little pulmonary blood flow, frequent findings of well-formed valve leaflets despite being fused, lack of arterial collaterals to the pulmonary circulation, a right ventricle that can vary in size, rare associations to extracardiac anomalies and anatomical similarities to newborns with critical pulmonary valve stenosis [91].

Abnormalities of coronary artery are as common as 70% at angiography [65, 93-95] however associated extracardiac anomalies are uncommon.

If left untreated, with closure of the ductus arteriosus in the first few days of life, survival will no longer be possible.

1.5.1.9 Double-Inlet Ventricle

Another form of cyanotic CHD is double-inlet ventricle. This condition is characterised as the presence of two atrioventricular valves with either, only one ventricular chamber, or a large dominant ventricle accompanied by a miniscule opposing ventricle [96-98].

The incidence is reported to be in around 1.25% of infants with congenital heart disease [99].

During the early stages of embryologic life, the atrioventricular canal unites with the ventricular part of the primitive heart tube, which during later stages this becomes the left ventricle. Blood passes from the ventricular portion of the primitive heart tube to the bulbus cordis, which later contributes to the right ventricular development. An arrest or a defect in interventricular septation results in a double-inlet single left ventricle with an underdeveloped right ventricle outflow chamber [98].

Trabeculation characteristic as well as the anatomical position of the atrioventricular valves can be used to identify whether the chamber of a single ventricle heart is most like a left or a right ventricle.

Infants with single ventricle and pulmonary atresia are cyanotic at birth. The amount of pulmonary blood flow provided by ductus arteriosus, persistent aortopulmonary collaterals, or bronchial circulation determins the degree of cyanosis in these patients. Patients with severe pulmonary stenosis are comparable to those with pulmonary atresia and their clinical presentation is determined by the amount of pulmonary blood flow, which is limited by pulmonary stenosis or raised pulmonary vascular resistance. In the absence of pulmonary stenosis and falling foetal pulmonary vascular resistance, there will be a gradual increase in pulmonary blood flow, which can ultimately cause congestive heart failure. A small number of patients with pulmonary blood flow limited to about double the systemic blood pressure may do well for several years and despite their noticeable cyanosis, have a normal growth.

The commonest form of double-inlet ventricle is a single left ventricle with L-transposition of the great arteries and accounts for the 74% of autopsied cases [98]. In this form, aorta arises from a minute leftward right ventricle, pulmonary artery usually ascends posteriorly, mitral valve is on the right side and the tricuspid valve to the left. *Holmes* heart is are and another form of double-inlet, single left ventricle without transposition with pulmonary stenosis.

1.6 Cardiac Physiology

1.6.1 Physiology of the developed Heart

1.6.1.1 Contraction:

Mammalian *cardiomyocyte* Contraction is a broadly studied subject [100-102]. Depolarisation of cardiomyocyte membrane initiates with an action potential. This prompts the entry of Ca^{2+} in the cardiomyocyte through L-type *calcium channels*. As a positive feedback result, Ca^{2+} is further released from the *sarcoplasmic reticulum* (SR) into the *cytoplasm*. Once there is a rise in the free intracellular Ca^{2+}, Calcium ions bind to *troponin-C*. Troponin-C is then attached to the contractile filaments of *actin* and is part of a regulatory complex. Upon the binding of Ca^{2+} to troponin-C, tropomyosin disengages from the actin-binding site. This facilitates the actin to bind the myosin ATPase placed on the *myosin* head. This results in hydrolysis of ATP that provides energy for a conformational change to take place in the *actin-myosin complex*. All these changes lead to a movement between the myosin head and the actin, in a way that the actin and myosin filaments slide past each other thus shortening the length of the sarcomere, resulting to cell contraction. Then the intracellular Ca^{2+} is shifted back to the SR or released outside the cell via the sodium/calcium exchanger (NCX). Upon the reduction of intracellular Ca^{2+} concentration, tropomyosin returns on the actin; which results in termination of contraction and ultimately the cardiomyocyte relaxes [102].

A large number of proteins that play a major role in cardiac physiology are Ca^{2+} sensitive. These include classical *protein kinases C* (PKC α, β, and γ), *ryanodine receptors* (RyR), *SR Ca^{2+} ATPase* (SERCA), and *phospholamban* [103, 104]. It has been described that disturbed Ca^{2+} handling can be responsible for contractile dysfunction such as heart failure and myocardial infarction [103].

1.6.1.2 Metabolism:

In a healthy heart, the main source of energy is delivered by fatty acids. This accounts up to 80% of the entire energy (**Figure 11**). The rest of the energy is supplied from lactate and glucose oxidation [105].

Figure 11 Cardiac energy production
(Reillustrated image from Stanley 2005[106].

Normally, metabolism of myocardium is aerobic. Throughout normal physiological conditions, mitochondrial oxidative phosphorylation supplies more than 95% of the ATP production in the heart. Mitochondrial oxidative phosphorylation provides energy from electron transfer. These are transferred by dehydrogenation reactions that generate *nicotinamide adenine dinucleotide hydrogen (NADH)* and *flavine adenine dinucleotide dihydrogen ($FADH_2$)*.
NADH and $FADH_2$ are primarily produced via the fatty acid β-oxidation pathway, the citric acid cycle (or Krebs cycle), and to a lesser extent from the pyruvate dehydrogenase reaction [106]. Approximately 60-70% of the ATP produced is used for the contraction. The remaining 30-40% is used by the sarco(endo)plasmic reticulum calcium-ATPase (SERCA) and other ion pump transport [106]. Figure 12 illustrates a summary of interactions between different myocardial metabolic pathways.

Figure 12 Different pathways of myocardial metabolism.
CPT-I= carnitine palmitoyltransferase-1; FAT= fatty acid transporter; G 6-P= glucose 6-phosphate; GLUT= glucose transporter; MCT= monocarboxylic acid transporters; PDH= pyruvate dehydrogenase; TG= triglycerides.
(Reillustrated image from Lopaschuk *et al*, American Heart Association [107])

As demonstrated in Figure 12, throughout the fatty acid metabolism, a fatty acid transporter (FAT) facilitates the entry of fatty acids to the cardiomyocytes. When the fatty acids have been transported across the SR, they become activated by esterification. Then, in the mitochondria, fatty acid β-oxidation occurs, which leads to the production of $NADH^{+}$, $FADH_2$, and acetyl-CoA. Acetyl-CoA is then degraded during the citric acid cycle and results in ATP production. During the carbohydrate metabolism, glucose enters the cell, which then converts to glucose 6-phosphate and degrades to *pyruvate*. In aerobic conditions, Pyruvate converts to *Acetyl-CoA* in the mitochondria. During the citric acid cycle, acetyl-CoA gets degraded, and this leads to energy production [106]. It is worth noting that *Creatine phosphate* is another source of energy for cardiomyocytes In addition to ATP [106].

1.6.2 Physiology of the Developing Myocardium

1.6.2.1 Changes in Myocardial Structure During Postnatal Development:

The structural and morphological changes to myocardium after birth have been extensively studied in animal models [108].

After birth, the body rapidly grows. In accordance to this, the heart also needs to grow in weight and size in order to comply with increasing demands of a rapidly growing body. Johnson *et al* showed that the weight of rat's heart increased 5-fold in the first 11 days after birth [109]. Hyperplasia in heart ceases 4 days after birth and from this point onwards heart's growth is a result of hypertrophy rather than hyperplasia [110, 111]. This transition from hyperplastic to hypertrophic growth is caused by a blockage of further mitosis [112].

The heart of a neonate in comparison to an adult contains more water, more non-contractile proteins and less collagen. This results in a less compliant neonatal myocardium when compared to an adult myocardium [113, 114]. Moreover neonatal cardiomyocytes exhibit disorganised myofibrils that are mainly found at the cell periphery. With age, myofibrillar organisation occurs, making the function of myocyte more efficient by allowing extracellular Ca^{2+} to enter the cell and rapidly target the contractile proteins to allow cell contraction [115].

Intracellular Ca^{2+} stores in the neonatal cardiomyocytes are not fully developed, and as a result, the immature myocardium requires a higher extracellular Ca^{2+} concentration to optimise its contractility [116]. In rat experimental models, it was discovered that, under normal conditions the neonatal mitochondrial uptake of Ca^{2+} is higher compared to the adults [117].

1.6.2.2 Postnatal Cardiac Development and Metabolism:

Fisher and co-workers demonstrated higher oxygen consumption in neonatal lamb cardiomyocytes when compared to foetal and adult cardiomyocytes [118]. This mirrors the changes in cardiac output for these particular age groups, with increased cardiac pump function associated with cardiac development [119].

The developing heart undergoes a metabolic transformation as it changes from an anaerobic intrauterine to an aerobic extrauterine environment. After birth there is an increase in fatty acid metabolism whilst glycogen and lactate concentrations declines [120]. Neonatal cardiomyocytes have higher glycogen content when compared to the adult cardiomyocytes, which declines remarkably between 7th and 14th day post birth, indicating increasing reliance on glycolysis. The tolerance to the anaerobic environment during embryogenesis begins to decrease after birth.

In the embryonic phase, the mitochondrial conversion of fatty acids to energy is noticeably lower than that of an adult. The embryonic development of the heart takes place in an environment that is relatively hypoxic and during this period, the embryo relies heavily on glycolysis for ATP production [121]. Glycolysis requires less oxygen to produce ATP and therefore it is favourable to the immature heart muscle [121].

Neonatal cardiomyocytes have a lower ability of aerobic ATP production comparing to the adult cardiomyocytes. As the mitochondrion matures, aerobic and oxidative metabolism capacity intensifies [122]. Several enzymes that are associated with the citric acid cycle and respiratory chain have been found to be low in activity in neonatal hearts, but as the cardiomyocytes adapt to the aerobic environment, the activity of these enzymes surges [123]. Throughout the postnatal period, the mitochondria continue to develop and increase in number and volume until they reach the maximal adult number [124].

Cardiomyocyte mitochondrial Ca^{2+} capacity changes throughout postnatal development. Bassani *et al* showed an increased capacity of Ca^{2+} within the mitochondria via the Ca^{2+} uniporter in neonatal rat cardiac mitochondria. They also reported a decline in this capacity after the first 2 weeks of birth [117].

1.6.2.3 Variance in Antioxidant Status During Developmental Period:

Das *et al* demonstrated in their study that the antioxidant defence system in pigs undergoes developmental changes in the early phases of neonatal growth with minimal changes afterwards [125]. However, so far this finding has not been demonstrated in human or any other animal studies. Work on rat heart indicates changes that continue to take place beyond post-neonatal stage. Xanthine oxidase activity increases from birth to 16th week of age in the rat heart [126]. Xanthine oxidoreductase activities in adult rats are found to be significantly higher when compared to the new-born [127].

As well as adult cardiomyocytes, there is an upregulation of neonatal antioxidants during stressful conditions. Following exposure of cultured neonatal heart cells to non-lethal doses of hydrogen peroxide, a significant upregulation of catalase activity and mRNA expression was generated without having effects on SOD or GPx levels/activity [128].

1.6.2.4 Postnatal Development and Mechanism of E-C Coupling:

In rat models following birth, the ventricular contractility index (an indicator of contractility which implicates wall stress, cell shortening and the force of contraction) substantially increases in the first week of life. In addition to this, as the heart develops and matures, the ventricular function noticeably increases [119]. The contractile response to a low Na^{+} concentration differs from day-to-day in the first week of life indicating rapid developments to postnatal contractile structures [129]. Numerous studies have shown that throughout the myocardial development, the E-C coupling mechanism can change dramatically [130-132].

Wibo *et al* reported an increase of more than double in number of RyRs per gram of tissue between 2 and 30 days of age in conjunction with proliferation of the SR membranes [130]. In rat cell culture model, cardiomyocytes have been shown to have a different RyR cellular arrangement, density and Ca^{2+} transit. The major differences are between day 1 and day 4 [133]. However, since this has not been studied in vivo, one cannot confidently conclude its application to E-C coupling during development of an intact and working heart cell.

In the first few weeks after birth, the SR and surface membrane systems are not yet fully developed. As a result the release and reuptake of Ca^{2+} in E-C coupling mechanisms in the neonate is less active. Neonatal SRs, however, have an intact Ca^{2+} store, indicating that the neonatal SR is primed with Ca^{2+} that is not fully released in response to a stimulus [134]. The T-tubular network continues to develop for weeks following birth and initiates to extend from the periphery to the interior of the cell, 7 days after birth [135].

There is also a significant difference in action potentials of neonate and adult hearts [136]. Neonatal rats have longer action potential plateau duration with an increased delay prior to repolarisation when compared to adult rats [137]. Studies of developing human cardiomyocytes indicate that with age, L-type Ca^{2+} channels increase, whilst T-type Ca^{2+} channels decrease. These studies also showed that NCX mRNA and protein levels were higher in foetal hearts when compared to adult, whereas SERCA2a protein expression increased with age, with a maximal expression in adulthood [138, 139]. There is also a lower density with different inactivation of outward K^+ channels in neonatal cardiomyocytes [136]. A significant reduction in action potential duration was observed between one day old and mature cardiomyocytes, which corresponded to a significant increase in K^+ currents and the mRNA expression of the involved K^+ channels [140].

Higher levels of T-type channels and NCX imply that; sarcolemmal Ca^{2+} handling proteins play a key role in the sarcolemmal Ca^{2+} entry via the abundant T-type channels (rather than few L-type channels) in neonatal hearts [138]. It is proposed

that structures such as SR and T-tubules along with Ca^{2+} handling proteins in immature human heart become equivalent to those of an adult at about three weeks of age [139].

Table 1 is a summary of some of the physiological differences between adult and paediatric myocardium [141].

Table 1 Physiological Difference Between Paediatric and Adult Myocardium and Potential Impact of These Differences on Ischaemia Tolerance of the Paediatric Heart

	Paediatric	Adult	Potential Impact on Ischaemia Tolerance in the Paediatric Heart
Preferred substrate for adenosine triphosphate production	Glucose	Fatty acids	Increase
Glycogen content	High	Low	Increase
Insulin sensitivity	Impaired	Normal	?
Calcium handling (intracellular)	Impaired	Normal	?
Calcium sensitivity	Increased	Normal	Decrease?
Antioxidant defence	Low	High	Decrease
5' nucleotidase	Low	High	Increase
Catecholamine sensitivity	Low	Normal	?
Ischaemic preconditioning	Absent	Present	?

Doenst T, Schlensak C, Beyersdorf F. Cardioplegia in paediatric cardiac surgery: do we believe in magic? *Ann Thorac Surg* 2003;75(5):1668-1677 [141]

1.6.3 Cardiac metabolism during hypoxia:

Principally *hypoxia* or oxygen deficiency is defined as an imbalance between oxygen supply and demand. Heart energy is mainly provided in the form of adenosine triphosphate (ATP). As discussed above, most of this ATP production is via oxidative phosphorylation in the mitochondria.
Hypoxia can lead to ionic changes in cardiomyocyte that can result in accumulation of H^+; i.e. acidosis [105, 142].

Throughout hypoxia, there is a decline in ATP production that derives from mitochondrial oxidative phosphorylation. This is caused by a fall in carbohydrate and fatty acid oxidation. Cardiac metabolism thus switches to an anaerobic mode and induces the accumulation of pyruvate that is converted to lactate. Increase in intracellular lactate and protons instigates *acidosis* in the cardiomyocyte [142]. Proton increase also induces an intracellular increase of Na^+ and Ca^{2+} through the Na^+/H^+ and Na^+/Ca^{2+} exchangers [105]. Moreover, accumulation of intracellular proton can obstruct the activity of contractile proteins by competing with calcium [143, 144].

The mitochondria generate the vast majority of cellular energy production as ATP. This ATP synthesis is associated with *reactive oxygen species (ROS)* production. Energy production is mainly by oxidisation of glucose, pyruvate and NADH, which is oxygen dependent. During hypoxia, ATP availability becomes limited due to the build-up of non-esterified fatty acid levels, acidosis, and Krebs cycle blockade. Furthermore, hypoxia induces increased levels of mitochondrial NADH which can stimulate the augmentation of the $NADH/NAD^+$ ratio [105]. In vivo studies have shown that myocardial mitochondria increase in number when chronic hypoxia is induced [145].

There is evidence that with ischaemia-reperfusion, the myocardial metabolism can become disturbed, leading to the opening of *mitochondrial permeability transition pore (mPTP)* during this injury [146].The mitochondrial permeability transition pores (mPTP) are non-selective pores, permeable to very small

molecules (<1.5 kDa). They open in certain strict conditions such as abnormal elevation of calcium levels [146]. This opening of pores can lead to two major consequences. The first one is *uncoupling*, that may lead to ATP hydrolysis rather than synthesis. The second consequence is mitochondrial *swelling* [146]. Whether the ischaemia-reperfusion injury is reversible or irreversible, largely depends on the extent of the mPTP opening [147, 148]. All these imply that reactive oxygen species (ROS) production in cardiomyocytes is ultimately affected by hypoxia-induced stress.

ROS are highly reactive molecules that are present in all cells. They contain an unpaired outer shell of electron that makes them highly reactive. In cardiomyocyte, reactive oxygen species are thought to be vital for intracellular signalling during hypoxia [149]. Mitochondrion is the principle source of ROS and ROS are formed as by-products during the oxidative phosphorylation.
The four main ROS are:

peroxynitrite ($\cdot OONO^-$) hydrogen peroxide (H_2O_2)
hydroxyl radical ($\cdot OH^-$)
superoxide anion ($\cdot O_2^-$)

Peroxynitrite which is a result of superoxide and nitric oxide, is known to have a strong detrimental and toxic effect on the myocardium [150-152]. Via Fenton chemistry, hydrogen peroxide may be converted into hydroxyl radical. Once the hydroxyl radicals are formed, they can then react with the cell nucleic acids, lipids and proteins and cause cell damage and even death [153]. Hydroxyl radicals have lipid peroxidation characteristics, which is basically an oxidative degradation of lipids. This can affect the ion channels and pump function in cardiomyocytes and lead to contractile injury [153].
Superoxide anions can be produced in the electron transport chain and can then be converted into hydrogen peroxide by *superoxide dismutase (SOD)*. Depending on the cellular positioning of SOD and their related metal cofactor, three different SOD isoforms exist [154]:

CuSOD (SOD1) which is intracellular
MnSOD (SOD2) which is located in the mitochondria
ZnSOD (SOD3) which is extracellular

Although ROS is fundamentally produced by mitochondrion, there are other sources to ROS. These additional sources are primarily *NADPH oxidase (NOX)*, *nitric oxide synthase (NOS)* and *xanthine oxidase (XO)*. XO catalyses the oxidation of hypoxanthine to xanthine and then further to uric acid [155-158]. This oxidation process will lead to production of hydrogen peroxide and superoxide, which can then result in cell injury [159].
NOS are composed of three isoforms [160]. Of all three NOS isoforms, endothelial NOS (eNOS) is the one that as a result of limited bioavailability of tetrahydrobiopterin (BH4) can easily get uncoupled and ultimately lead to superoxide production [161].
ROS, as well as being harmful to the cell, have been shown to have an important role in signalling. ROS are known to act as second messenger downstream of certain growth factor stimuli such as TGF or PDGF [162].

Production of ROS and the mechanisms that could counterbalance their production is summarised in Figure 13.

Figure 13 Simplified scheme of ROS production.
$O_2^{\cdot}$**=**Superoxide, ·OH=hydroxyl radical, $\cdot OONO^-$=peroxynitrite, GPx=glutathione peroxide, NOS=nitric oxide synthase, NOX= NADPH oxidase, XO=xanthine oxidase, SOD=superoxide dismutase
(Image reillustrated from Giordano 2005[153])

A crucial element of the hypoxic response is the production of a transcription factor called *hypoxia inducible factor 1 (HIF-1)* and is the most important factor during a hypoxic state [163]. It is composed of a constitutively expressed β subunit (HIF-1β), and an inducible α subunit (HIF-1α) [163]. Throughout normoxic conditions, HIF-1α is a target for prolyl hydroxylation by HIF prolyl-hydroxylase, which utilises oxygen as a co-substrate. This results in HIF-1α degradation by an ubiquitin ligase and then destruction by the proteasome. However, in hypoxic circumstances, HIF prolyl-hydroxylase is inhibited and HIF-1α protein expression is therefore stabilised, which leads to HIF-1α accumulation in cytoplasm and ultimately in the nucleus [164].

Cardiomyocyte Kinases are also affected in hypoxic conditions. Several signalling pathway specific protein kinases are controlled during hpoxia. These protein kinases are; *mitogen activated protein kinase*, *Phosphatidylinositol 3'-kinase*, *Protein kinases C*.

Mitogen activated protein kinases (MAPK):

MAPK are serine/threonine-kinases. They play an important role in intracellular signalling. Some stimuli including hypoxia can activate MAPK in cardiomyocytes [165-169]. This has been demonstrated in cultured rat cardiomyocytes [170, 171].

Phosphatidylinositol 3'-kinase (PI3K):

PI3K can be activated by different stimuli such as receptor *protein tyrosine kinases (RPTK*) and *heterotrimeric G-protein-coupled receptors (GPCR)*. PI3K catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-tris-phosphate (PIP_3). This leads to the activation of 3-phosphoinositide dependent protein kinase-1 (PDK1), which in turn activates many proteins including *protein kinase B (PKB)* also known as *Akt*. Activation of

the PI3K/PDK1/Akt pathway is known to have a *cytoprotective* characteristic in all different cells [172].

A summary of the survival pathway mediated by the PI3K/PDK1/Akt axis is illustrated in Figure 14.

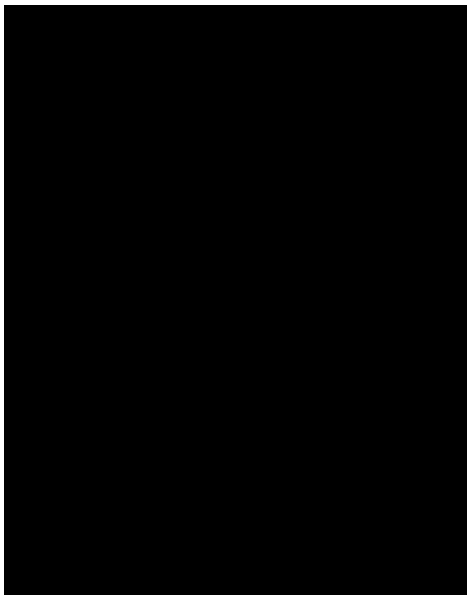

Figure 14 Simplified scheme of the PI3K/PDK1/Akt pathway.
(Reillustrated image from Katso 2001[172])

Protection of cardiomyocyte against hypoxic/ischaemic conditions via PI3K/Akt pathway has been demonstrated in various studies. This protection has been shown in vivo as well as in vitro [173-176]. Ravingerova demonstrated that in isolated rat heart, during ischaemia-reperfusion injury, there was an increase in the size of infarct when PI3K was pharmacologically inhibited. This indicates a possible role for PI3K in cell survival [174].

There are also reports that *heat shock protein 90 (HSP90)* is associated with the survival pathway mediated by the PI3K / Akt axis [177]. In a separate study Wang *et al* demonstrated an increase in HSP90 levels in rat cardiomyocytes following hypoxic insult [177].

Protein kinase C (PKC):

PKC is also a family of serine/threonine-kinases. It plays a significant role in the signalling events associated with myocardial contractions [178]. PKC has numerous isoforms. These isoforms are classified into four categories: conventional (α, β and γ), novel (δ, ε, η and θ), atypical (ζ and λ) and lastly the fourth category is composed of μ and ν isoforms [178, 179].

1.6.3.1 Ischaemia-Reperfusion Insult and Vulnerability of the Developing Cardiomyocyte compared to adult:

Although a general belief is that the normal neonatal myocardium is more resistant to ischaemia-reperfusion injury than mature adult myocardium [180, 181], this topic remains controversial and there are conflicting reports in the literature regarding this. Doenst *et al* reported a greater neonatal myocardial ischaemia tolerance versus adult [141]. Additionally, there are other reports suggesting that in comparison to an adult cardimyocytes, the postnatal developing cardiomyocytes demonstrate a greater resistance against injurious effects of I/R [129, 137, 182-185]. Although there are some theories to explain this, the exact mechanism behind this remains unclear [185]. Some of these theories are: lower energy demand, higher tissue glycogen and glycolysis [186], greater anaerobic capacity and also variances in Ca^{2+} handling and ROS production in postnatal [185]. There appear to be strong associations between the superior postnatal resistance against detrimental effects of I/R and differences in the ROS generation and/or removal upon reperfusion [129, 184, 187]. This is supported by the findings of de Jong *et al* when they showed significantly higher levels of xanthine oxidoreductase in adult rat cardiomyocytes when compared to the new-born [127]. This implies that the lower activity of a younger heart may contribute to its greater resistance to ischaemia.

By contrast to the above studies, there are some reports indicating that the postnatally developing myocardium is more susceptible to reperfusion injury when compared to the adult heart [188-190].

Wittnich *et al*. reported that adult pigs could tolerate 50% longer period of ischaemia before they developed hypercontracture when compared to the newborn pigs [191]. There are also reports that neonatal cardiomyocytes are more susceptible to Ca^{2+} overload [192]. Other reports have suggested that during ischaemia, neonatal heart accumulates more H^+ with a lower capacity to buffer the intracellular pH [190]. Finally there are also some reports that in the clinical setting, the paediatric myocardium is more susceptible to ischaemic injury than adult during cardiac surgery [193].

More studies may need to be carried out on this topic to explain these conflicting results.

1.6.4 Response to stress, inflammation and myocardial damage

There are numerous studies that have investigated cardiac insults as a result of myocardial ischaemia-reperfusion injury. The sudden oxygen supply to any organ including myocardium, which has been subjected to ischaemia/hypoxia, could lead to production of harmful components such as reactive oxygen species, hormones and cytokines [194, 195]. Re-oxygenation injury is therefore one of the adverse effects of treatments available for acute myocardial infarction. This is caused by restoration of blood flow through the coronary arteries in order to improve the survival of patients with cardiovascular diseases [196]. It also has been described that myocardial injury can be worsened if the reperfusion is associated with cardioplegic arrest [197].

1.6.4.1 Stress response:

Stress response can be defined as body's reaction to a stressor, which can result in production of some inflammatory factors. In a clinical setting, cortisol is considered to be an important stress indicator [198, 199]. Stimuli such as infection, burn, pain, ischaemic-reperfusion injury, or even mental stress, can activate the hypothalamic-pituitary-adrenal (HPA) axis. This can lead to the release of corticotropin releasing hormone (CRH). CRH can then trigger the release of adrenocorticotropic hormone (ACTH) by acting on the anterior part of the pituitary gland. ACTH in turn stimulates the adrenal cortex to release glucocorticoids such as cortisol [200]. Cortisol activates the hepatic gluconeogenesis process, which then lead to an increase in blood glucose level. Cortisol has also immunosuppressive and anti-inflammatory properties. By inhibiting genes that are involved in coding for several cytokines including IL-2, IL-6 and IL-8, cortisol supresses cell mediated immunity [201]. In addition to this, cortisol diminishes eosinophilia as well as inhibiting many functions of leukocytes and immune accessory cells triggering anti-inflammatory response.

There are suggestions that glucocorticoids have cardioprotective properties [202, 203]. Barzilai *et al*. demonstrated a reduction in mortality rate following acute myocardial infarction in patients who were treated with hydrocortisone [202]. In a separate study, Valen *et al*. showed in a rat experimental model that glucocorticoid can have

cardioprotective properties [203]. There are reports that in adult patients who underwent open-heart surgery, plasma cortisol levels were shown to be elevated [194].

1.6.4.2 Inflammation:

Inflammation is part of the body's protective reactive complex response to a noxious stimulus with the intention to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult [41]. Many studies have been carried out in inflammatory response in cardiovascular diseases which involve molecular interactions between cardiomyocytes, endothelial cells and leukocytes [204]. Normally, vascular endothelial cells oppose bonding to leukocytes, yet in certain conditions such as atherosclerosis this physiology is disturbed. The adhesion of leukocytes to the endothelial wall leads to increased expression of inflammatory mediators such as cytokines [204]. Cytokines are important components of the inflammatory response. Cytokines can be subdivided to pro-inflammatory and anti-inflammatory. The pro-inflammatory cytokines promote inflammation, whereas anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines resulting in inflammation reduction [205].

It is a well-known fact that cardiac surgery with the aid of cardiopulmonary bypass (CPB) is associated with a higher acute inflammatory response. This may affect the myocardial function and post-operative recovery [206]. In the heart, interleukin 6 and 8 (IL-6 and IL-8) play a pro-inflammatory role while IL-10 is described to have an anti-inflammatory effect [206]. IL-10 has also been described to be cardioprotective in the context of myocardial ischaemia-reperfusion injury in mice [207]. Injured myocardium can be a source of pro-inflammatory cytokines such as tumour necrosis factor α (TNF α) and IL-6. [208]. Moreover, ischaemic treatment has been shown to induce IL-6 production in adult cardiomyocytes [209]. IL-8 is another pro-inflammatory cytokine and has also shown to be released by the ischaemic myocardium. IL-8 can have a critical role in neutrophils recruitment [210].

1.6.4.3 Myocardial Injury:

Myocardial injury is damage to the heart tissue, which could lead to myocardial dysfunction and can occur after ischaemia-reperfusion. Reperfusing the myocardial tissue following a period of ischaemia can lead to myocardial cell necrosis [196]. Moreover, it is believed that during the reperfusion of ischaemic myocardium, ion homeostasis is lost through calcium sequestration in the cells with disturbances in the Na^+/K^+ and Na^+/Ca^{2+} pumps. Reoxygenation may result in accumulation of intracellular hydrogen ion causing acidosis, which can be lethal for the cells. Acidosis can also prevent the contractile apparatus to function normally and can lead to cardiac failure [196]. Myocardial injury may be diagnosed by raised levels of serum troponin which is normally located inside cardiomyocytes [211]. Troponin has three components: C, I and T. They are integral to the function of striated muscles and exist as a complex with actin and tropomyosin on the thin filament of the contractile apparatus [212]. Troponin-C binds calcium and regulates the activation of thin filaments during contraction. Cardiac troponin-I is an inhibitory component of contractile interaction and troponin-T binds to tropomyosin [212]. Although both cardiac troponin T and I are used in clinical settings to assess myocardial injury, there are some evidences indicating that cardiac troponin-I is a more sensitive and specific marker for assessment of myocardial injury. It is also argued that the levels of Troponin-I show minimal alteration in the presence of renal failure [213, 214].

1.7 Cardiopulmonary Bypass

It is over 60 years since John Heysham Gibbon for the first time performed a successful open-heart surgery using the heart-lung bypass machine.
On 6th of May 1953 in Philadelphia, J.H. Gibbon, Jr, MD, of the Jefferson University Medical Centre, closed a large secundum atrial septal defect in an 18-year-old lady with the aid of cardiopulmonary bypass (CPB)[215]. Cardiopulmonary bypass and open heart surgery has since majorly developed.

It may not be possible to perform cardiac surgery without having to stop the heart and bypassing the pulmonary circulation. Cardiopulmonary bypass machine, featuring a pump to act as a substitute for the function of the heart and a gas exchange device, the "oxygenator," to act as an artificial lung can facilitate this. This will allow to transiently ceasing the circulation to the patients' heart and lung and to suspend respiratory and cardiac activity, providing a safe and controlled environment for cardiac surgery.

The basic mechanism of the CPB machine is as follows: Venous blood is drained from the right atrium or large veins through a plastic cannula into the reservoir. From there it is pumped through the oxygenator back into the patients' arterial system through a different plastic cannula that inserts into either aorta or another large artery. As the blood transitions through the oxygenator, the partial pressure of carbon dioxide is reduced and the oxygen content is raised (Figure 15).

Figure 15 Typical configuration of a cardiopulmonary bypass circuit.
(Image from Practical Approach to Cardiac Anaesthesia, 4th Edition, Hensley *et al*.2008 [216])

1.7.1 Oxygenators:

Since the application of extracorporeal circulation and CPB, the term oxygenator has been used, however *blood gas exchange device* is a more correct description. This is because its function is to regulate oxygen, carbon dioxide and nitrogen. It also facilitates the administration of anaesthetic gases during CPB.

Some oxygenators are designed to have special coating or treatments that reduce the biochemical cascades affiliated with systemic inflammatory response (SIR). This is aimed to minimise CPB-induced SIRs [217-220]. The other important considerations in oxygenator design include flow related characteristics, aiming to decrease blood cell trauma as well as minimising the priming volume and device surface area. The heat exchanger of the oxygenator must also be able to proficiently transfer heat energy and efficiently thermoregulate.

The mechanisms of gas exchange between the native lung and oxygenator are different. In the lungs, red blood cells pass through pulmonary capillaries in single file,

reducing the distance for O_2 diffusion. Therefore except in the case of extreme exercise or severe lung disease, the rate of oxygen transfer is not limited by diffusion[24]. In the oxygenator however, since the distances are much greater, to achieve an adequate gas transfer, it requires a significant difference in partial pressures of gases to exists between the gas and blood phases of oxygenators, even under normal operating conditions [221].

Gas exchange is based on Fick's Law of Diffusion:

$$\text{Volume of gas diffused} = \frac{\text{Diffusion coefficient} \times \text{Partial pressure difference}}{\text{Distance to travel}}$$

By using gas blenders, oxygenators can ventilate with different percentages of O_2 (21-100%). Gas blender allows blending medical grade air and oxygen, to maximise the driving pressure difference for O_2 diffusion [221].

1.7.2 Myocardial Protection and Cardioplegia Delivering System:

A major priority during cardiac surgery is myocardial protection during the ischaemic period. This can be achieved by using a solution referred to as "cardioplegia" that induces a state of hibernation of the heart.

The term *myocardial protection* indicates the possibility of myocardial injury of some sort. In cardiac surgery this is largely exhibited by injury as a result of ischaemia-reperfusion [221]. However, one must not forget that myocardial protection does not exist in isolation; and it is important to consider non-cardiac organ protection as well.

Myocardial Injuries as a result of ischaemia-reperfusion can be classified into two separate categories, reversible and irreversible. Myocardial oedema and a temporary decline in cardiac function are the manifestation of reversible myocardial injuries that resolve with time without significant long-term sequelae. Conversely, irreversible myocardial injuries implicate myocardial apoptosis or necrosis that can lead to release of some cardiac biomarkers such as creatine phosphokinase (CK) or troponin (Tn) into the circulation as well as electrocardiographic changes. Irreversible injuries result in permanent abnormalities of cardiac function [221].

During the early days of cardiac surgery, there were reports of successful surgery when the heart was arrested for a very short period [222-226]. However as the cardiac surgery advanced, more complex cardiac cases were considered for surgery, which required a longer period of cardiac arrest. This extended phase of unprotected cardiac ischaemia resulted in greater myocardial injury and poor outcomes. Later hypothermia was considered as a measure to allow a lengthier period of safe cardiac arrest. However it became apparent that after an extended period of time, despite hypothermia, the myocardium is subject to injury and quite often resulted in "stone heart" [221].

In 1955 and 1957, Melrose induced cardiac arrest by using potassium citrate. Although somewhat primitive (a 0.5% solution of potassium citrate was injected directly into the aortic root after a cross-clamp had been applied), it did effectively stop the heart [227]. Unfortunately, the Melrose solution in isolation did not provide adequate myocardial protection, and its popularity weakened.

The extensive work of Hearse and Braimbridge *et al* at St. Thomas' Hospital in London on cardioplegia solution ultimately led to the developed the "St. Thomas Solution 1 (STH 1)" which provided reliable cardiac arrest with good myocardial protection [228, 229]. Their solution was a major advancement in the field of myocardial protection and gained a substantial international popularity. In the late 1970s Follett and Buckberg described more complex strategies of cardioplegia delivery, including refinements in temperature and route, which further advanced the efficacy of cardioplegia [230, 231].

Currently, countless choices can be considered when choosing a myocardial protection strategy and there are more than 150 different available cardioplegia solutions [232] . The primary differences in composition of cardioplegia solutions aim to address the mechanisms involved in ischaemia-reperfusion injury [233-237].

Although cardioplegic arrest has become the mainstay of myocardial protection, the utility of intermittent fibrillation and deep hypothermic circulatory arrest, also needs to be appreciated.

Cardioplegia can be delivered in many different ways; antegrade, retrograde, or both either intermittent or continuous.
It is important to bear in mind that, there is no such thing as "best" cardioplegia strategy for all circumstances and practice environments; however, a working knowledge of the range of cardioplegia options that are available is useful so that the surgeon can provide optimal clinical care.

1.7.2.1 Cardioplegia Composition:

Cardioplegia is a solution that utilises a rapid cardiac arrest in diastole and provides myocardial protection from ischaemia and reperfusion. Potassium with concentrations ranging from 10 to 40mmol/L is the most commonly utilised agent to deliver cardiac arrest [228, 230, 231, 238].
Cardioplegia composition has been described as either "intracellular" (low sodium and calcium concentrations) or "extracellular" (higher concentrations of sodium, calcium, and the addition of magnesium)[221].
Furthermore, cardioplegia can be divided into two subtypes; crystalloid cardioplegia and blood cardioplegia. A variety of supplementary electrolyte and pharmacologic mediators with different concentrations can be added to each of these subtypes.

1.7.2.2 Temperature and myocardial protection:

Despite numerous cardioplegia solutions there are disagreements on the type of cardioplegia that should be used despite attempts by many studies to address this issue [12, 239-243]. Additionally, there is no unified consensus in the literature regarding different cardioplegia temperatures and their myocardial protection efficacy [244, 245]. Although some studies have demonstrated the superiority of one cardioplegia

temperature over another [246, 247], other studies have been unable to demonstrate a difference [248, 249].

One possible reason to these conflicting conclusions may be the fact that, different patients may benefit from different cardioplegia regimen. Given the variation in cardioplegia composition, temperature, duration and delivery routes in different studies, makes comparison and conclusions very difficult and problematic.
In our institution, Ascione *et al.* evaluated the differences in myocardial protection afforded by warm and cold intermittent antegrade blood cardioplegia in patients with hypertrophic hearts undergoing aortic valve surgery [250]. Authors reported less myocardial injury and less ischaemic stress in patients receiving cold cardioplegia. Caputo *et al.* also from our institution, demonstrated no difference in post-operative renal impairment between the cold and warm cardioplegia in paediatric cases undergoing cardiac surgery [251]. Fan *et al*. in a meta-analysis of 41 randomised controlled trials, which included 5879 patients, reported that comparison between warm and cold cardioplegia for myocardial protection during cardiac surgery resulted in, similar incidences of clinical events, significant improvement in postoperative cardiac index and reduction in postoperative enzyme release [244].

1.7.2.3 Myocardial Protection and Preservation for Neonates and Infants:

It is important to appreciate that cardiac surgery on the neonate and infant heart differs from cardiac surgery on the adult heart in a number of ways. The coronary arteries and myocardium are, for the most part, normal in patients with congenital heart disease. This can provide a uniform delivery of cardioplegia. However, the surgeon is faced with a heart that could be in different phases of development. This matter is of significant importance to the surgeon since during various stages of myocardial maturity, some transitions take place that influence the ability of the myocardium to withstand periods of ischaemia and injury. The transitional point is not exactly known but it is thought to occur in the first year of life or possibly within the first 3 months of life [221].

Some believe that the normal neonatal myocardium is more resistant to ischaemia-reperfusion injury than mature adult myocardium [180, 181]. However, this added resistance does not seem to be present in the hearts of cyanotic neonates and children [5, 252]. Therefore myocardial protection techniques must be tailored with respect to the age of the patient, pathology and complexity of the procedure to ensure the best outcomes.

1.7.2.4 Cardioplegia and hypothermia in neonates and infants:

There are disagreements over optimal strategy for myocardial protection in the neonate and infant [253]. However, the most frequently practiced principles to protect myocardium during paediatric cardiac surgery are; hypothermia to minimise myocardial metabolic activity and delivery of cardioplegia to temporarily cease the cardiac contractile and electrical activity [221].

Primarily, administration of cardioplegia is for cessation of all contractile and electrical activity of the heart that results in a significant reduction of energy consumption, even at normothermia [254].

In neonates and infants, hypothermia alone may provide a significant amount of myocardial protection. In fact some authors have suggested that in certain conditions, cardioplegia may not even be necessary [141, 255]. This theory is supported by reports suggesting equal or superior myocardial protection with hypothermia alone when compared to hypothermia combined with cardioplegia [256-259]. It is noteworthy however to point out that these studies were carried out in very low systemic temperatures (15°C or less). Reports from other studies at a higher temperature steadily show that hypothermia with the addition of cardioplegia is advantageous [12, 260, 261].

Figure 16 [141] plots benefit from cardioplegia against the non-cardioplegic establishment of the same temperature (i.e., topical cooling, blood perfusion etc.) for myocardial function during ischaemia. This chart indicates that the benefit of cardioplegia in the neonatal period appears to be directly related to myocardial temperature.

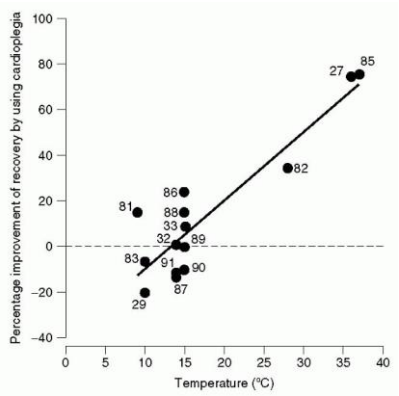

Figure 16 Benefit of cardioplegia over establishment of the same temperature by a non-cardioplegic method (i.e., topical cooling, blood perfusion, perfusion with crystalloid buffer) as a function of myocardial temperature during ischaemia. A positive value indicates that cardioplegia was better than the non-cardioplegic method. A negative value indicates that the non-cardioplegic method was better. (Doenst T, Schlensak C, Beyersdorf F. Cardioplegia in paediatric cardiac surgery: do we believe in magic? Ann Thorac Surg 2003;75(5):1668-1677)

1.7.3 Haemofilters:

Excessive fluid accumulation is a phenomenon that often neonates and infants encounter through exposure to CPB [262-264]. This oedema develops not only in the periphery but also in vital organs such as the brain, heart, intestine, and lungs [265]. Different methods can be used to remove or minimise this excess fluid in order to increase the haematocrit levels to satisfy oxygen demands. Utilisation of ultrafiltration, which removes excess fluid while still on CPB, is one of the ways to achieve this. However, this can be very challenging in neonates. In this group of patients the circuit is very small and as a result any removed fluid may have to be replaced again to maintain adequate reservoir level. Therefore zero-balance ultrafiltration (Z-BUF) during CPB may be a more practical method of fluid removal. In this method, volume of fluid exchange corresponds to the volume filtered. Studies have shown, Z-BUF to be beneficial as it can reduce postoperative blood loss, decrease time to extubation, and remove considerable amount of circulating TNF, IL-10, myeloperoxidase, and C3a [266, 267]. Modified Ultrafiltration (MUF), which was described by Elliott and Naik is another form of ultrafiltration that is performed after the completion of CPB while the aortic and venous cannulae are still in place. This appears to be a more effective form of ultrafiltration [268, 269]. MUF has the advantage of filtering the patient's extracellular blood volume as well as the residual circuit volume, which results in greater haemoconcentration [268]. Both conventional and MUF are effective in filtering inflammatory mediators from the circulation [270]. However, MUF has been shown to be more effective in haemoconcentration and

improving both, pulmonary compliance, and ventricular functional recovery [268, 271, 272].

1.7.4 Prime volume:

Prime volume is the amount of fluid necessary to fill both arterial and venous limbs of a cardiopulmonary bypass circuit. Prime volume also includes the fluid required for the reserve volume in the venous reservoir that prevents air entry to the arterial segment of the circuit.

In adults, the prime volume is about 30-35% of patients' total circulatory volume. However, in children, particularly neonates and infant, even the smallest possible priming volume exceeds patients total blood volume. Therefore, using non-blood prime solutions in these cases is not a feasible option.

Principally, an ideal priming solution must have a similar pH, tonicity and electrolyte arrangement to the patients' plasma. Tonicity is the most important matching characteristic for the priming volume since a tonal mismatch will result in red cell breakdown (haemolysis) leading to fluid shifting from extracellular to the intracellular compartment. This fluid shift is observed with hypotonic solutions and may occur in any tissue or organ, but those organs that are particularly susceptible to this fluid shift are the brain and lungs [273].

Despite variation in practice amongst different units in the United Kingdom, the most frequently used crystalloid to prime cardiopulmonary bypass circuit is the Hartmann's solution [273].

1.7.5 Effects of pH and temperature on oxygen delivery:

Blood is predominantly composed of plasma and red blood cells (RBCs). The fractional volume of the blood composed of RBCs, is referred to as 'haematocrit'. Blood carries oxygen in two forms:

1. Dissolved in plasma and RBC water (~ 2% of the total)
2. Reversibly bound to haemoglobin (~ 98% of the total)

At a physiological PO_2 (~100 mm Hg), only a small amount of oxygen is dissolved in the plasma since oxygen has a low solubility. However the physically dissolved form of oxygen becomes substantial once the PO_2 is raised.

Blood pH as well as body temperature can affect oxygen-carrying capacity and delivery.

An increase in blood carbon dioxide results in decreased pH (acidosis), which reduces the affinity of haemoglobin for oxygen. The oxygen dissociates from the Hb molecule, shifting the oxygen dissociation curve to the right. Therefore, more oxygen is needed to reach the same haemoglobin saturation level as when the pH was higher. A similar shift in the curve also results from an increase in body temperature. Temperature is associated with both the delivery and consumption of oxygen and is therefore vital to effective CPB. A drop of 10°C in temperature slows the rate of metabolic reactions by about half. In addition hypothermia increases blood viscosity and shifts the oxygen-haemoglobin dissociation curve to the left, both of which decrease oxygen delivery/release. As blood temperature falls, gas solubility rises and the partial pressure of carbon dioxide decreases (PCO_2 decreases 4.4% for every °C drop in temperature) resulting in alkalosis.

1.7.6 Acceptable haemodilution:

Some degree of haemodilution may be beneficial as blood viscosity is reduced, improving microcirculatory flow [274]. In general, acceptance of a degree of haemodilution during CPB, the use of autologous priming, collection and processing of shed mediastinal blood and the return of residual pump blood at the end of CPB can

all lead to a decrease in allogenic blood transfusions with their consequent risks and uncertain risk/benefit profile [275, 276].

HCT is the main determinant of the oxygen-carrying capacity of blood.
Factors affecting the HCT during CPB include [273]:

- Patient size;
- Preoperative haemoglobin concentration/HCT;
- Pre-CPB blood loss;
- Pre-CPB fluid administration;
- CPB prime volume; and
- Urine output

Theoretically, minimum acceptable HCT should meet the oxygen delivery *(DO_2)* required to match systemic O_2 consumption *(VO_2)*. However, DO_2 is not controlled by reflex mechanisms, but by the perfusionist. During CPB whole body DO_2 is a function of pump flow and arterial oxygen content, the latter being primarily determined by the HCT. The major determinants of VO_2 are temperature and level of anaesthesia.

Oxygen consumption (VO_2) can be calculated using the Fick equation:

$$VO_2 = Q(C_{(a-v)})\ O_2$$

VO_2 = minute oxygen consumption (ml/minute)
Q = cardiac output (l/minute)
$(C_{(a-v)})\ O_2$ = $1.34 \times Hb + P_{(a-v)}0_2$

where 1.34 is the haemoglobin oxygen content at 100% saturation (ml/g), Hb is the haemoglobin concentration (g/l) and $P_{(a-v)}O_2$ is the arterio-venous oxygen partial pressure difference (mmHg).

Indices of adequate total perfusion include pH, lactate and SvO_2 (haemoglobin oxygen saturation in venous blood). A low SvO_2 during CPB indicates an imbalance between DO_2 and VO_2 and requires a change in perfusion conditions. The SvO_2 value should always also be interpreted in the context of core temperature. The solubility and haemoglobin binding affinity of oxygen increases with hypothermia, whilst organ

metabolic demand decreases, resulting in increased SvO_2 if perfusion is adequate.

1.7.7 Triggers of CPB induced organ damage:

The key mechanisms in causing organ damage associated with CPB are [273]:

- The activation of a systemic inflammatory response (SIRS), which is an inevitable consequence of CPB
- Hemodilution and reduced blood viscosity, mainly at the onset of CPB, resulting in alterations in the distribution of blood flow to organs and flow characteristics of blood through capillary networks
- Ischaemia/reperfusion injury to heart, lungs and organs supplied by the splanchnic circulation
- Laminar rather than pulsatile flow, although the significance of this remains controversial.

1.7.7.1 Ischaemia–reperfusion injury (IRI):

IRI is the term used to describe the cellular injury that occurs on resumption of normal perfusion to an organ after a period of relative or complete ischaemia. This has already been discussed earlier, but in summary; during the ischemic period intracellular calcium accumulates due to the failure of ATP-dependent cellular pumps. On reperfusion, intracellular calcium levels further increase secondary to oxidative dysfunction of sarcolemma membranes. This cellular and mitochondrial calcium overload ultimately induces cardiomyocyte death by hypercontracture and opening of the mitochondrial permeability transition pores (mPTP) on the inner mitochondrial membrane. Opening of the mitochondrial PTP channels during early reperfusion (they remain closed during the ischemic period) inhibits the mitochondrial membrane potential, uncoupling oxidative phosphorylation, which being essential for ATP production, results in ATP depletion and cell death. Large quantities of oxygen free radicals are generated on reperfusion of ischaemic tissue. The oxygen free radicals, if present in sufficient concentration, overwhelm endogenous scavenging mechanisms

and cause further intracellular injury. Oxygen free radicals also exacerbate arachidonic acid metabolism and the production of leukotrienes and thromboxanes, promoting aggregation, transmigration and activation of neutrophils to further compound the injury. Neutrophils are the key final mediators of IRI by the production of toxic chemicals generated during the metabolism of oxygen and by the secretion of proteolytic enzymes released from granules stored in their cytoplasm.

The rapid breakdown of ATP during ischaemia results in generation and accumulation of the metabolite hypoxanthine. The enzyme *xanthine dehydrogenase* normally metabolises hypoxanthine. Under conditions of ischaemia followed by rapid reperfusuion, *xanthine dehydrogenase* is converted to *xanthine oxidase* as a result of the higher availability of oxygen. This oxidation results in molecular oxygen being converted into highly reactive superoxide and hydroxyl radicals. Excessive nitric oxide produced during reperfusion reacts with superoxide to produce the potent free radical peroxynitrite. These radicals attack cell membrane lipids, proteins and DNA, causing further cell damage or death.

1.7.8 Hyperoxic CPB, it's pitfalls and the concept of normoxic CPB

Despite the routine use of hyperoxic CPB in many cardiac centres throughout the world, this practise is probably unnecessary, since a PaO_2 of 400–500 mmHg results in only a trivial increase of the oxygen content compared with a PaO_2 of 100–150 mmHg [277].

Usually CPB is commenced at a PaO_2 in the range of 300–400 mmHg in cyanotic population undergoing corrective cardiac surgery. This can have a cytotoxic effect as a result of exposure to a high partial pressure of oxygen. In addition there are reports that hyperoxic CPB, can impair glucose regulation which can result in hyperglycaemia [278], diminish peripheral perfusion and skeletal muscle oxygenation [277].

One of the proposed strategies to minimise reoxygenation injury is the reduction of PaO_2 during CPB. Studies have shown a direct correlation between the partial pressure of oxygen (PaO_2) and reoxygenation injury [10, 254, 279], which can be avoided by controlling the PaO_2 to the normoxaemic range (80–100 mmHg) during CPB [10, 279].

In 1995, Buckberg *et al* [254] proposed the concept of ''surgical reoxygenation injury of cyanotic myocardium''. They reported that unintended rapid reoxygenation of cyanotic cardiomyocyte during a routine CPB may result in an oxygen radical-mediated myocardial injury highlighting post-CPB cardiac dysfunction. Del Nido and co-workers in a clinical setting demonstrated the relevance of such myocardial injury by detecting increased lipid peroxidation in pre-ischaemic myocardial biopsies taken from the right ventricle of children with tetralogy of Fallot, showing oxidative injury at the start of CPB [8].

Morita and colleagues in two separate studies using animal models of acute hypoxaemia confirmed the role of reoxygenation injury in post-CPB myocardial dysfunction and addressed the significance of controlling PaO_2 at the initiation of CPB for cyanotic hearts [18]. They also later confirmed that a period as short as 5 minutes of uncontrolled reoxygenation was enough to cause myocardial damage by establishing that controlling the PaO_2 during blood cardioplegia from 5 min after the initiation of CPB had no protective effect on the myocardium [279].

The first clinical evidence of reoxygenation injury was reported by Allen *et al* followed by [14] Bulutcu *et al* [280]. They reported a significant depletion of the antioxidant reserve capacity in cyanotic infants following sudden reoxygenation with hyperoxic CPB. Allen *et al*. showed that this hyperoxic injury could be significantly minimised by utilising a lower PaO_2 (FiO_2 of 0.21) at the onset of CPB [14]. Although however, they pointed out that an FiO_2 of 0.21 can still results in a PaO_2 of 140–155 mmHg, which is substantially higher than the PaO_2 of 80–100 mmHg. Similarly, Bulutcu *et al*. demonstrated that antioxidant depletion induced by reoxygenation can be significantly ameliorated by initiating CPB using FiO_2 of 0.21 (PaO_2, 90–110 mmHg) [280]. These clinical reports indicate the need to assess oxygenation status by PaO_2 and not FiO_2, in order to reliably achieve normoxia. By delivering nitrogen to the oxygenator it is possible to achieve a PaO_2 of 80–100 mmHg in the CPB priming fluid with FiO_2 of 21%. [281].

The first real clinical evidence of myocardial reoxygenation injury as a distinct entity from ischemia/reperfusion injury was reported by Modi *et al*. [5]. They studied twenty-nine paediatric patients undergoing cardiac surgery. Twenty were cyanotic and 9 were acyanotic. All patients underwent at least 30 minutes of hyperoxic CPB before ischemic cardioplegic arrest. They assessed troponin I at 1, 10, and 30 minutes of CPB. They reported an increase in troponin I levels with time in both groups however the rate of increase was greater in cyanotic population by about 3 times.

It is important however to point out that higher oxygen levels during CPB should be considered if there is a requirement for a period of low flow or deep hypothermic circulatory arrest for neurologic protection [282]

2 - Method and Study Design

2.1 Hypothesis and study proposal:

Based on the earlier work of Modi *et al.* [5](discussed in the previous chapter), we hypothesised that by reducing the oxygen tension to the patient's own levels (normoxic) on initiating cardiopulmonary bypass, we may attenuate the detrimental effects of cardiopulmonary bypass on myocardial function, and re-oxygenation injury. We proposed to carry out, in a prospective randomised clinical study, a comparison between normoxic (70-100 mmHg) and standard (relatively hyperoxic) (150-200 mmHg) CPB for the repair of congenital cyanotic heart disease.

2.1.1 Definitions:

Cyanosis was defined as a pre-operative diagnosis of a cyanotic cardiac condition plus oxygen saturation of 90% or less at all times. "Normoxia" referred to a pump prime, the pO_2 of which is matched to the pO_2 of the patient (i.e., normoxic for the patient). "Hyperoxia" referred to a pump prime prepared to the current "best practice" protocol, which has a pO_2 relatively hyperoxic for a cyanotic patient.

2.2 Method:

Parents/guardians of cyanotic patients who were undergoing surgical repair of their congenital cyanotic heart disease that were suitable for this study were approached and an informed consent was abtained. They were asked to sign a consent form, which was approved by Ethical Committee. The patients were then randomly allocated to either normoxic (70-100 mmHg) or standard (relatively hyperoxic) (150-200 mmHg) CPB. Randomisation was accomplished by card allocation on the night before the surgery. The surgical team, except for the perfusionists, were blind to the treatment allocation.

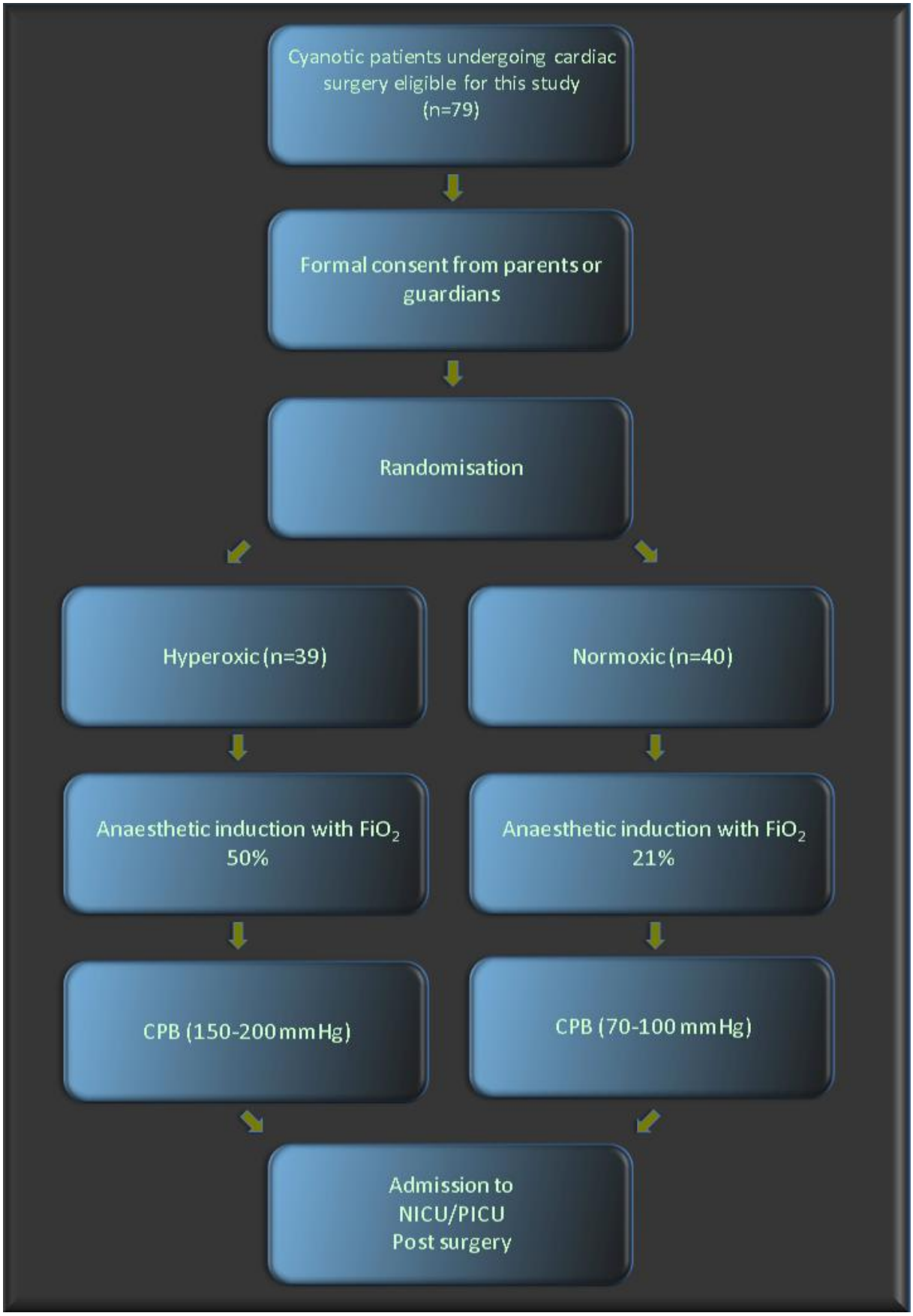

Figure 17 Summary of study design

Seventy-nine cyanotic patients undergoing corrective cardiac surgery between January 2003 and March 2007 at the Bristol Royal Hospital for Children were randomised to receive either controlled normoxic (70-100 mm Hg) or hyperoxic (150–200 mm Hg) CPB. All patients were in a stable condition without preoperative respiratory or inotropic support.

Treatment allocations, stratified by age (<6 months vs ≥6 months), were generated by computer in advance of starting the study, using block randomisation with varying block sizes. Allocation details were concealed in sequentially numbered, opaque sealed envelopes. After consent was obtained the night before the operation, a patient was randomly assigned from the next numbered envelope.
After the operation, all patients were admitted to the Paediatric Intensive Care Unit (PICU) and were managed according to unit protocols [283, 284] by intensivists and paediatric cardiologists who were also blinded to the treatment allocation.

2.2.1 Anaesthetic and surgical techniques:

All operations were performed using CPB with ascending aortic cannulation and bicaval venous cannulation. Anaesthetic techniques were standardised for all patients and followed the Check List:

Induction: The normoxic group induction of anaesthesia was started with a fraction inspired oxygen (FiO_2) of 0.21 and in the hyperoxic group with an FiO_2 of 0.50. Continuous pulse oximetry was used in every patient from the start of anaesthesia.
Sevofluorane or midazolam (200 to 500 mcg/kg); pancuronium 200mcg/kg iv; fentanyl 5 mcg/kg iv; commence fentanyl infusion – 25 mcg/kg/hr; dexamethasone 0.5 mg/kg iv (maximum 8mg); run isofluorane as required; reduce fentanyl infusion to 10 mcg/kg/hr after 1 hour.

Pump drugs:
Midazolam 500 mcg/kg; pancuronium 200 mcg/kg.

Pre-CPB:

Fentanyl infusion as above; reduced to 10 mcg/kg/hr after 1 hour bypass. Fentanyl infusion increased to 15 mcg/kg/hr

Post-CPB:

Morphine/fentanyl infusion; midazolam +/- vecuronium until ready for extubation; phenoxybenzamine or SNP if necessary.

2.2.2 Cardiopulmonary bypass (CPB) Techniques:

Normoxic (Iso-oxic) CPB for the normoxic group: As discussed earlier, currently, the common practice is to deliver to the cyanotic patients, the same levels of oxygen that one would deliver to the acyanotic ones[1, 2, 8]. We have developed a novel and simple cardiopulmonary bypass strategy of controlled oxygenation at the Bristol Royal Hospital for Children[3] with a hypothesis that this could reduce the risk of ischaemic-reperfusion injury. For this purpose, the CPB circuit is set up and primed usually with a homologous red blood cell/albumin prime or a clear prime, depending on the patient's haemoglobin level. Just before the initiation of CPB, medical nitrogen is delivered to the gas exchange device (oxygenator) via a bacteriologic filter (0.2 μm) at a rate of between 100 and 200 mL/min while the prime is circulated at approximately 1000 mL/min. An in-line pO_2 monitor measures' the pO_2 of the prime (in air this will equilibrate at roughly 150 mm Hg). Using this technique, we are able to reduce the pO_2 of the prime fluid to less than 100 mmHg and actually match that of the patient's own arterial pO_2 levels. Before the establishment of CPB, the prime pO_2 is confirmed using a point of care blood gas analyser, and the in-line pO_2 monitor is calibrated. CPB is initiated in an "iso-oxic" manner and the pO_2 levels of the arterialised blood are adjusted accordingly throughout the CPB. So by using air and minimum required O_2 we maintain a SaO_2 at preoperative levels as much as possible (SaO_2<90%). Hence we just use an initial N_2 flush and then we started CPB in air (F_iO_2: 0.21), which keeps the pO_2 at preoperative levels. We then slowly raise the pO_2 so that termination of CPB occurs with an arterial pO_2 of between 100 and 110 mm Hg.

Standard CPB for hyperoxic group: Run at 100% O_2 to keep SaO_2>95%.

2.2.2.1 Cardioplegia:

Cold blood (4-6^{o} C) St Thomas' I based cardioplegic solution (4:1 dilution of homologous or autologous blood/St Thomas' I crystalloid cardioplegia) were used for myocardial preservation following aortic cross clamping in both groups, with the following composition (mmol/L): 16$MgCl_2$, 2$CaCl_2$, 20KCl, 147NaCl, 1.0 procaine HCl. Additional cardioplegia was administered approximately after each 20 minutes of aortic cross-clamping.

2.2.3 Sample Size and Statistical Analysis:

The sample size was calculated on the basis of previous similar studies carried out at our institution in which statistically significant reductions in troponin-I release (effect size 0.48) and in 8-isoprostane (effect size 0.94) were found with 55 and 42 patients, respectively[283]. With one preoperative and five postoperative measurements, a sample size of 40 per group has more than 90% power to detect effect sizes of 0.5 or more for both markers at the 5% level of statistical significance (2-tailed) assuming a correlation of 0.7 between preoperative and postoperative values and between postoperative measures.

Continuous outcomes are summarised as an arithmetic mean and standard deviation if normally distributed or as a geometric mean or median and interquartile range if skewed. Categorical data are presented as actual counts and percentages.

Skewed measures were log-transformed to achieve normality and the results were back transformed to the original scale.

Biochemical markers measured at multiple time points were analysed with a mixed regression model. All the markers had skewed distributions and were analysed on the logarithmic scale.

An overall estimate, pooled over all time points, is reported. Effect sizes (also known

as ‘strength of ssosiation’), which indicate the relative magnitude of the differences between means, are reported as mean differences (if normally distributed) or as ratios of geometric means (if skewed) with corresponding 95% confidence intervals (CIs) and *p* values. According to Cohen’s guidelines, an effect size of 0.2 or less is considered small and above 0.8 is large. Effect sizes between small and large are considered medium.

The baseline results are not reported in the charts since this study was designed to assess the effect of normoxic versus hyperoxic CPB. However baselines blood samples were taken to make sure that there were no diffences between the two groups pre-treatment.

Since only scores of differences betwen two groups (normoxic and hyeroxic) were measured, analysis of variance (ANOVA) had no role in the statistical analysis for this study.

2.2.4 Data Collection:

The collection of data included: clinical information (i.e. age, sex, body surface area, preoperative medications); pre and postoperative echocardiographic findings; preoperative oxygen saturation, routine pre- and postoperative blood tests (i.e. haematocrit, creatinine and electrolytes); operative procedural information (i.e. type of operation, CPB and cross-clamp duration), inotropic or vasodilator support, intubation time, death, renal failure and postoperative hospital stay (see Data Collection Sheet in appendix).
All these parameters were defined according to the standard written protocols used in the Intensive Care Unit at the Bristol Children Hospital. We later analysed the effect of normoxic versus hyperoxic CPB in single and biventricular patients separately.

2.2.5 Proposed Biochemical Tests:

Troponin I (as a myocardial damage indicators), 8- isoprostane (a marker of oxidative stress), alpha glutathione S-transferase [aGT] (a liver enzyme which raised levels may suggest liver injury), serum S100 (raised levels may indicate possible CNS insult) plus general inflammatory markers including complement activation C3a, serum cortisol, interleukin [IL] -6, -8, and -10.

The blood samples were taken at: anaesthetic induction, 10 and 30 minutes after initiation of CPB, plus 10min, 4 hours and 24 hours post cessation of CPB.

All blood samples were taken from the arterial lines.

Each time 1ml of blood sample was used for arterial blood gas (ABG) analysis. Furthermore, 1ml of blood in EDTA bottle and 3ml of blood in Lithium Heparin bottle were stored. The EDTA and Lithium Heparin bottles were placed in a container filled with ice during transport to laboratory. The samples were then centrifuged at 4 degrees Celsius for 15 minutes at 4000 revolutions per minute to isolate the plasma.

The plasma samples were then stored in special containers using pipet and were immediately frozen in liquid nitrogen. They were then stored in the freezers that were set at -80 degrees Celsius on level 7, Bristol Heart Institute. Plasma from the EDTA bottle was used for C3a measurement and plasma from Lithium Heparin bottle was used for the analysis of other aforementioned tests.

Mr Mark Ginty, an experienced laboratory technician with previous experience in similar blood sample analysis, measured the systemic and organ specific injury markers in Bristol Heart Institute's laboratories.

2.3 Primary and secondary endpoint:

Primary end points are the release of troponin I and 8-isoprostane as a measurement of myocardiac cell damage and oxidavtive stress. Secondary endpoints include; release of markers of whole body inflamtory response (complement activation C3 alpha, interleukin 6, 8 and 10), stress response (cortisol), cerebral injury (protein S100) and hepatic insult (alpha-glutathion S-transferase).

2.4 Ethical approval, research governance and indemnity:

The Ethics Committee had issued an approval for this study. United Bristol Healthcare Trust Research and Development (R&D) sponsored the trial.

2.5 Storage of records:

Study records (both hardcopy and electronic) were retained in a designated and secure location on level 7, Bristol Heart Institute during the conduct of the trial and after completion. All source documents will be retained for a period of 5 years following the end of the study. Where trial-related information is documented in the medical records, the records are identified with a "Do not destroy before dd/mm/yyyy" label, the date being 5 years after the last patient was recruited for the trial.

2.6 Research Governance Statement:

This study was conducted in accordance with the Research Governance Framework for Health and Social care and Good Clinical Practice.

3 - Biochemical Assays

3.1 Markers of systemic and organ specific injury:

3.1.1 Cardiac Troponin-I (cTn-I):

Historically, the diagnosis of acute myocardial injury has been via history taking and physical examination in conjunction with 12-lead electrocardiography (ECG). Later, serial creatine kinase isoenzyme (CK and CK-MB) measurements, and determination of lactate dehydrogenase (LDH) isoenzyme ratios were used to diagnose myocardial injury. However their popularity declined due to limitations such as lack of accuracy in patients with renal impairment [285, 286].

One set of assays focused on proteins from the troponin complex. Three troponin subunits regulate muscle contraction by modulating the calcium-dependent interaction of actin and myosin: the tropomyosin binding subunit T, the calcium-binding subunit C, and the actomyosin ATPase inhibiting subunit I. Troponin-I exists in three isoforms: slow-twitch, fast- twitch, and cardiac. Cardiac troponin-I (cTn-I) is a 26.5 kDa isoform of the muscle subunit, and is genetically and structurally distinct from that produced in extracardiac muscle [287, 288].

Cardiac troponin-T (cTn-T) and cTn-I correlate with acute myocardial injury in the general population, both turning positive within 6–8 h and remaining so for at least 4–7 days [289, 290]. Spurious cTn-T elevations, similar to CK-MB, have been identified in patients with renal failure [291, 292]. Cardiac troponin-I however, has been shown to be specific for myocardial damage in the setting of renal failure, hypothyroidism, rhabdomyolysis, skeletal muscle injury, perioperative period, burns and cocaine intoxication [293-298]. Due to its high specificity, cTn-I appears to be ideally suited for the detection of myocardial injury and myocardial necrosis in complex clinical situations [290]. Measurement of cTn-I could simplify the care of patients by definitively excluding or confirming the presence of acute ischaemic myocardial injury [290].

3.1.1.1 Principles Of The Procedure:

Access Immunoassay System®; Beckman Instruments Inc, Troponin I ELISA was used for the measurement of troponin levels in our samples. The assay is based on enzyme immunoassay (ELISA) technology using horseradish peroxidase as a label. 25 μL of plasma is required and the hook effect is avoided by using 2-step assay format (30 min + 30 min). The substrate contains both 3,3', 5,5' tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in one bottle format with greatly enhanced stability and sensitivity. Increasing absorbance is monitored at 450 nm (Figure 18).

Results calculation is by calculating the mean of the duplicate absorbance determinations. A standard curve on graph paper is then constructed by plotting the mean absorbances of the six standards (ordinate) against the corresponding cTnI concentration (abscissa). The best fitting curve will then be drawn.

Figure 18 Serum Tn-I Measurement
(Image from www.beckmancoulter.com)

3.1.2 8-Isoprostane:

The isoprostanes are from eicosanoids family and are produced via tissue phospholipids oxidation with oxygen radicals. Isoprostanes can materialise as artefacts in plasma or tissue samples that have suffered prolonged oxidative degradation during improper storage. Under normal conditions small levels of isoprostanes may be detectable in the plasma and urine; however, the levels will significantly rise by oxidative stress [299].

One of the isoprostanes, 8-isoprostane (8-*iso* Prostaglandin F2α), has been particularly reported to have biological activity. This prostaglandin compound that belongs to the F2 isoprostane class is formed in vivo by the free radical-catalysed peroxidation of arachidonic acid [300]. It has very strong renal and pulmonary vasoconstrictor properties [301] and is associated to causative mediators of hepato-renal syndrome and pulmonary oxygen toxicity [302]. Assessment of in vivo oxidative stress by measurement of 8-Isoprostane has emerged as one of the most reliable approaches that provides an essential tool to investigate the role of oxidative stress in the pathogenesis of human disease [303]

Elevated levels are found in heavy smokers and can also be an indicator of antioxidant deficiency [304]. 8-Isoprostane can also be used to assess the sample integrity for lipid-containing specimen such as serum, plasma, and whole cell preparations [305]. Plasma of healthy individuals may contains modest amounts of 8-isoprostane (40-100 pg/ml) that can increase with age [306].

3.1.2.1 Principle of the method:

Cayman Chemical™ 8-Isoprostane EIA kit was used for this study.
This assay is based on the competition between 8-isoprostane and an 8-isoprostane- acetylcholinesterase (AChE) conjugate (8-Isoprostane Tracer) for a

limited number of 8-isoprostane-specific rabbit antiserum binding sites. Because the concentration of the 8-Isoprostane Tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-Isoprostane Tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-isoprostane in the well. This rabbit antiserum-8- isoprostane (either free or tracer) complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of 8-Isoprostane Tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well during the incubation.

Figure 19 Schematic of the ACE™ EIA
(Diagram from www.caymanchem.com)

3.1.3 Interleukin-6 (IL-6):

Unlike many cytokines interleukin-6 is both produced by and has effects on a broad spectrum of cell types. The range of activities include both growth promotion and inhibition, regulation of immunoglobulin and acute phase protein gene expression and induction of differentiation [307].

Human interleukin-6 ((h)IL-6) is a 212 (precursor), 184 (mature) amino acid protein with a molecular weight of 23–30 kDa (determined by SDS PAGE) which is N-linked glycosylated [307, 308]. Numerous cell types produce IL-6 including: B

and T cells and cell lines, monocytes and monocyte lines, fibroblasts, endothelial cells, keratinocytes, bone marrow stromal cells and several tumour cell lines. The IL-6 receptor is a heterodimeric molecule consisting of an 80 kDa IL-6 binding protein and a 130 kDa accessory molecule, which is required for the high affinity form (1×10^{-11} M) that can transduce signals. The IL-6 receptors have been found to be on a wide range of cells including B and T cells, monocytes, myelomas, hepatocytes, hepatomas and astrocytomas up to 10 000 per cell [308-310]. A soluble circulating form of the 80 kDa IL-6 binding protein is able to form a complex with IL-6 which can then associate with the membrane bound 130 kDa molecule resulting in signal transduction.

Investigations of IL-6 in vivo have demonstrated that its levels may alter in several phenomena. These include ischaemia-reperfusion experiments in some animal models as well as ischaemia-reperfusion injury post-transplant in human [311-313].

3.1.3.1 Principle of the method:

Amersham Interleukin-6 [(h)IL-6] Human, Biotrak ELISA System,GE Healthcare, was used for the measurement of plasma IL-6 in this study.

This immunometric assay is based on a solid phase ELISA, which utilises an antibody for (h)IL-6 bound on the wells of a microplate together with an antibody to (h)IL-6 conjugated to biotin and streptavidin-HRP detection. (h)IL-6 can be measured in the approximate range of 1-400 pg/ml (0.5-20 pg/well) in less than 4 hours.

This assay employs the quantitative 'sandwich' enzyme immunoassay technique. An antibody specific for (h)IL-6 antibody has been coated on the microplate provided in the kit. Samples are pipetted into the wells and the (h)IL-6, if present, is bound by the IL-6 immobilised antibody. A biotinylated antibody reagent is added to the wells and allowed to bind to any IL-6 bound by the

immobilised antibody in the first incubation. After washing away any unbound biotinylated antibody a streptavidin-HRP conjugated is added to the wells. Any IL-6, which was bound by both the immobilised and the biotinylated antibody during the first incubation, will be bound by the streptavidin conjugate. Following a wash to remove unbound conjugate, a substrate solution is added to the wells and colour develops in proportion to the amount of (h)IL-6 bound in the initial step.

In addition to the samples to be tested, a series of wells are prepared using known concentrations of the human IL-6 standard. A curve, plotting the optical density versus the concentration of the standard well is prepared. By comparing the optical density of the samples to this standard curve, the concentration of the (h)IL-6 in the unknown samples is then determined.

3.1.4 Alpha Glutathione S-Transferase (Alpha GST or αGT):

Hepatic reoxygenation injury has been demonstrated in experimental animal models of rats undergoing induced hypoxia for 60 minutes followed by 25 minutes of reflow [314]. It has also been shown that during reoxygenation of perfused rat liver, there is an increased oxyradical production leading to liver injury [315]. The alpha glutathione S-transferase (άGTs) are a group of cytosolic proteins that constitute from 2% to 5% of the soluble protein in hepatocytes [316].

In liver, άGTs is located in the hepatocytes whereas $_{pi}$GTs (πGTs) is confined to the intrahepatic bile duct cells [317-320]. This heterogeneous GST subclass distribution suggests that the isoenzymes have unique *in vivo* functions in different hepatic regions and that the detection of GST subclass levels in biological fluids would be of significant use in monitoring the integrity of specific hepatic regions.

A routine and a common way of to evaluate liver function are to measure the liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The disadvantage of these markers is that they are not distributed

uniformly throughout the liver, the periportal concentration being greater than the centrilobular [317]. In contrast, αGTs has been found to be equally distributed in both the centrilobular and periportal regions [321]. Centrilobular hepatocytes are very susceptible to damage in a variety of clinical conditions including allograft rejection [322], viral hepatitis [323], chronic active hepatitis [324], and hepatotoxicity [325].

The baseline level of άGTs in serum is very low, and as such it is easy to monitor any increases that may occur. άGTs is a very sensitive and specific biomarker of hepatocyte injury [326]. It is unaffected by muscle injury and other factors that can cause elevated transaminase levels [316]. An elevated άGT level indicates hepatocyte injury even when other markers are normal. A normal serum άGT level almost excludes acute hepatocyte injury [316].

3.1.4.1 Principle of the method:

Biotrin High Sensitivity Alpha GST EIA Enzyme Immunoassay kit was used for this test.
This is a quantitative enzyme immunoassay. The test procedure is based on the sequential addition of sample, enzyme-conjugate and substrate to microassay wells coated with anti-αGST. The resultant colour intensity is proportional to the amount of αGST present in the sample. The assay range is 62.5-2000ng/L.

3.1.5 Protein S100:

Hypoxia and reoxygenation are known as important mechanisms of cerebral injury. Sher and Hu demonstrated in an in vitro cell model that gradual reoxygenation after prolonged hypoxia improves neuronal survival compared with rapid reoxygenation and delays the manifestations of metabolic dysfunction [327]. Their findings are also consistent with the concept that a period of relative hyperoxia may contribute to hypoxia- induced neuronal injury. Stauton *et al* have also shown that hypoxemia– reoxygenation causes endothelial dysfunction

in intraparenchymal cerebral arterioles by impairing endothelium-dependent dilation of microvessels, which in turn may decrease oxygen delivery and increase neuronal injury [328].

S100, a 20 kDa glial-cell specific protein, was initially isolated from human brain [329]. However to date, based on structural and functional similarities, 20 different monomers of the S100 family have been described [330, 331]. The majority of the S100 proteins present in dimers form and their expressions are cell-specific. S100A1 and S100B are two S100 monomers that are highly conserved amongst different species [332]. They are identified as homo- (BB) and heterodimers (A1B) in glial cells of the central nervous system and in some certain peripheral cells such as melanocytes, adipocytes, Schwann cells and chondrocytes [333].

S100 is a useful biomarker in the management of patients with cerebral insult such as: head trauma, perinatal asphyxia, cardiac arrest and CVA [334-337]. It is considered to be one of the most specific and sensible markers of cerebral injury after cardiac surgery and is widely used in clinical trials [338].

3.1.5.1 Principle of the method:

The CanAg S100 EIA Kit was used to measure S100. This is a solid-phase, two-step, non-competitive immunoassay based on two mouse monoclonal antibodies specific for two different epitopes expressed in S100B. The assay determines both S100A1B and S100BB without cross-reactivity with other forms of S100. Calibrators and samples are incubated together with biotinylated Anti-S100B monoclonal antibody (MAb) S23 in Streptavidin coated microstrips. S100B present in calibrators or samples is adsorbed to the Streptavidin coated microwells by the biotinylated Anti-S100B MAb during the incubation. The strips are then washed and incubated with horseradish peroxidase (HRP) labelled Anti-S100B MAb S53. After washing, buffered Substrate/ Chromogen reagent (hydrogen peroxide and 3, 3', 5, 5' tetra-methylbenzidine) is added to each well and the enzyme reaction is allowed to proceed. During the enzyme reaction a blue colour will develop if antigen is present. The intensity of the colour is

proportional to the amount of S100B present in the samples. The colour intensity is determined in a microplate spectrophotometer at 620 nm (or optionally at 405 nm after addition of Stop Solution). Calibration curves are constructed for each assay by plotting absorbance value versus the concentration for each calibrator. The S100B concentrations of samples are then read from the calibration curve.

3.1.6 Complement 3 Alpha (C3a):

Activation of the classical, alternate, or lectin complement pathways can result in the production of the C3a anaphylatoxin [339]. C3a has been shown to be a multifunctional pro-inflammatory mediator. Thus, C3a is reported to increase vascular permeability, to be spasmogenic and chemotactic, and to induce the release of pharmacologically active mediators from a number of cell types [340].

C3a production in vivo may also initiate, contribute to, or exacerbate the inflammatory reactions seen in ischaemia-reperfusion injury, ischaemic heart disease, gram-negative bacterial sepsis, trauma, ARDS, post-dialysis syndrome, and several autoimmune diseases [341-343].

In blood plasma or serum, once formed, the nascent C3a anaphylatoxin is rapidly cleaved to the C3a-desArg form by the endogenous serum carboxypeptidase N enzyme [344]. Thus, the quantitation of C3a-desArg in plasma should yield a reliable measurement of the level of complement activation that has occurred in the test samples under investigation.

3.1.6.1 Principle of the method:

The BD OptEIA™ Human C3a ELISA Kit was used for the quantitative determination of Human C3a-desArg in our study.
The BD OptEIATM ELISA test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilises a monoclonal antibody specific for human C3a-

desArg coated on a 96-well plate. Standards and samples are added to the wells, and any C3a-desArg present binds to the immobilised antibody. The wells are washed and a mixture of biotinylated polyclonal anti-human C3a antibody and streptavidin-horseradish peroxidase is added, producing an antibody-antigen-antibody "sandwich". The wells are again washed and a substrate solution is added, which produces a blue colour in direct proportion to the amount of C3a-desArg present in the initial sample. The Stop Solution changes the colour from blue to yellow, and the wells are read at 450 nm

3.1.7 Interleukin-10 (IL10):

A systemic inflammatory response can occur following various conditions including ischaemia-reperfusion injuries [345]. A cascade of events including pro-inflammatory cytokine production can ultimately lead to multiple organ failure, and death [346].

IL-10 characteristically has inhibitory effects on pro-inflammatory cytokine production and physiology of individual cell types, which suggest that it could have potent anti-inflammatory activities in vivo.

A protective role of IL-10 in experimental endotoxemia has been demonstrated. Inhibition of TNF production in experimental endotoxemia was observed following IL-10 administration in baboons and humans [347, 348].

IL-10 was identified as the cytokine produced by the TH2 subpopulation of T cells that inhibit the synthesis of immunostimulatory cytokines by the TH1 cells. Human IL-10 can be produced by B and T cells, activated mast cells, macrophages monocytes and keratinocytes and is expressed as a noncovalently linked homodimer. Human IL-10 is a 178 amino acid protein with a molecular weight of 18 kDa with two N-linked glycosylation sites.
IL-10 has been shown to exhibit profound inhibitory effects on monocytes including down-regulation of major histocompatibility complex (MHC) class II

antigen expression, suppression of IL-1α and β, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF) and tumor necrosis factors alpha (TNFα) production. IL-10 has been shown to synergise with IL-2 and IL-4 to promote the proliferation of thymocytes. IL-10 has also been shown to act in conjunction with IL-3 and IL-4 to enhance the survival of mast cells.

Although IL-10 has been demonstrated to play an important role in several in vitro phenomena, the detection of IL-10 in vivo using bioassays or immunoassays has been difficult. Treatment of mice with anti-IL-10 antibodies resulted in the reversible depletion of Ly-1+ B cells, reduced serum IgM and IgA levels and attenuated antibody responses to hapten antigens. These mice also exhibited elevated levels of circulating IgG2a and IgG2b antibodies and TNFα. Mice lacking the IL-10 gene locus did not display any of the above effects, suggesting that immune complexes may have mediated the changes observed in anti-IL-10 treated mice. Agents that inhibit IL-10 production include IL-4 and IFNγ.

The suppressive effects of IL-10 on monocytes and TH1 cytokine synthesis suggest that IL-10 may have utility as a general suppressor of immune function. In endotoxin challenge models, IL-10 has shown efficacy in ischaemia-reperfusion and burn models [349, 350].

In addition, many strategies that are used to intervene in sepsis affect IL-10 production [351, 352] indicate an important role for this cytokine in controlling systemic inflammatory responses.

3.1.7.1 Principle of the method:

Amersham Biosciences© Interleukin-10 [(h)IL-10] Human, Biotrak ELISA System, GE Healthcare, was used.
This assay employs the quantitative 'sandwich' enzyme immunoassay technique.

An antibody specific for (h)IL-10 has been coated on the microplate provided in the kit. Samples are pipetted into the wells along with biotinylated antibody reagent, and the (h)IL-10, if present, is bound by both the immobilised antibody and biotinylated antibody. After washing away any unbound sample proteins and biotinylated antibody, a streptavidin-HRP conisjugate is added to the wells. Any (h) IL-10 which was bound by both the immobilised and the biotinylated antibody during the first incubation, will be bound by the streptavidin conjugate. Following a wash to remove any unbound conjugate, a substrate solution is added to the wells and colour develops in proportion to the amount of (h)IL-10 bound in the initial step.

In addition to the samples to be tested, a series of wells are prepared using known concentrations of the Baculovirus-derived human IL-10 standard. A curve, plotting the optical density versus the concentration of the standard well, is prepared. By comparing the optical density of the samples to this standard curve, the concentration of the (h)IL-10 in the unknown samples is then determined.

3.1.8 Interleukin-8 (IL8):

IL-8 which is primarily secreted by endothelial cells and monocytes was initially discovered in 1987 by Yoshimura by isolating it from human monocytes and distinguishing from from IL-1 by its chemotactic activity [353]. It is a heparin-binding protein precursor and a member of the CXC chemokine subfamily of cytokines. It contains 99 amino acids, however the mature functional protein, is comprised of 72 amino acids [354] Although *Chemokine Nomenclature Subcommittee of the International Union of Immunological Societies* renamed IL-8 to CXCL8, its approved gene symbol remains IL-8 [355]. Yet, IL-8 is also known by a variety of names, which describe some of its activities. These names include NCF (neutrophil chemotactic factor), NAP-1 (neutrophil activating protein), MDNCF (monocyte derived neutrophil chemotactic factor), or TCF (T-lymphocyte chemotactic factor).

IL-8 has pro-inflammatory properties and primarily facilitates the neutrophil activation and transendothelial migration from peripheral blood into the inflammation or infection sites in the tissue by interacting with CXCR1 and CXCR2 receptors [356, 357]. Once activated, both receptors couple to G protein to mediate phosphoinositide-hydrolysis, intracellular Ca^{2+} mobilisation, chemotaxis, and exocytosis [358, 359].

Reoxygenation of cultured hypoxic endothelial cells and monocytes induces the release of significantly elevated quantities of IL-8 [360, 361]. Neutrophil-mediated injury during reperfusion results when flowing neutrophils encounter activated endothelial cells expressing adhesion molecules that mediate an initial rolling and then firm adherence. During this process the neutrophils become activated, promoting chemotaxis into the ischaemic tissue [362-364]. Evidence suggests a role for IL-8 in the injury pattern seen after cardiopulmonary bypass. Elevated IL-8 levels are detected in the serum measured in coronary sinus blood after ischaemic cardiac arrest during heart operations requiring cardiopulmonary bypass [365, 366].

3.1.8.1 Principle of the method:

Amersham Interleukin-8 [(h)IL-8] Human, Biotrak ELISA System, GE Healthcare, was used for the measurement of plasma IL-8. This assay employs the quantitative 'sandwich' enzyme immunoassay technique. An antibody specific for (h)IL-8 is coated on the microplate provided with the kit. Samples are pipetted into the wells followed by incubation with biotinylated antibody reagent. If present, the (h)IL-8 is bound by the immobilised antibody and the biotinylated antibody. After washing away any unbound sample proteins and biotinylated antibody, a streptavidin-HRP conjugate is added to the wells. Any (h)IL-8 which was bound by both the immobilised and the biotinylated antibody during the first incubation will be bound by the streptavidin conjugate.

Following a wash to remove unbound conjugate, a substrate solution was added to the wells and colour developed in proportion to the amount of (h)IL-8 bound in the initial step.

In addition to the samples to be tested, a series of wells were prepared using known concentrations of the human IL-8 standard. A curve, plotting the optical density versus the concentration of the standard well, was prepared. By comparing the optical density of the samples to this standard curve, the concentration of the (h)IL-8 in the unknown samples was then determined (Figure 20).

Figure 20 Summary of IL-8 assay protocol
(Diagram from the products brochure available on www.gelifesciences.com)

3.1.9 Cortisol:

Cortisol (hydrocortisone, compound F) is a steroid hormone synthesised from cholesterol. It is the primary glucocorticoid produced and secreted by the adrenal cortex in response to adrenocorticotropic hormone (ACTH) and is the most abundant circulating steroid [367].

It is secreted with a circadian periodicity and peaks just prior to waking in the morning [368]. The production of glucocorticoids is increased by stress therefore

cortisol can be used as a biomarker of stress [369-371].

Cortisol binds to two intracellular receptors, the mineralocorticoid receptor (MR), and the glucocorticoid receptor (GR). Of the two receptors, the MR has the higher affinity for cortisol. This receptor will be almost completely occupied by cortisol at levels too low to activate the GR[370]. 11β-Hydroxysteroid dehydrogenase (Type 2) (11β-HSD2) converts cortisol to inactive cortisone. This enzyme is expressed predominantly in mineralocorticoid target tissues including kidney, colon, and salivary gland where it serves to protect the MR from glucocorticoid excess [372]. Individuals lacking this enzyme exhibit a syndrome known as *apparent mineralocorticoid excess* which features hypertension and hypokalaemia [369].

The enzyme 11β-HSD1 is a key regulator of intracellular glucocorticoid levels, catalysing the regeneration of cortisol from cortisone [373, 374]. Cortisol strongly promotes adipocyte differentiation; mature visceral adipocytes express high levels of the glucocorticoid receptor [373, 375].
Cortisol can be measured in many matrices including blood, faeces, urine, and saliva. Serum cortisol concentrations range from about 25-800 nM (9-300 ng/ml) and approximately 90-95% of the plasma cortisol is bound to proteins [376].

3.1.9.1 Principle of the method:

Access Immunoassay System, Beckman Coulter ™ ACETM EIA Kits was used for the quantitative analysis of cortisol levels in our study. This test kit operates on the basis of competition between the hormone conjugate and the cortisol in the sample for a limited number of binding sites on the antibody coated plate.
The sample or standard solution is first added to the microplate. Next, the diluted hormone conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites takes place. The plate is then washed removing all the unbound

material. The bound hormone conjugate is detected by the addition of substrate, which generates an optimal colour after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the wells of the samples against the standards with a microplate reader at 450nm or 650nm. The extent of colour development is inversely proportional to the amount of cortisol in the sample or standard. For example, the absence of cortisol in the sample will result in a bright blue colour, whereas the presence of cortisol will result in decreased or no colour development.

4 - Findings and Results

4.1 Results

Seventy-nine cyanotic patients undergoing corrective cardiac surgery were randomised to receive either controlled normoxic (70-100 mm Hg) or hyperoxic (150–200 mm Hg) CPB. (*Inclusion criteria*: All cyanotic patients who underwent congenital cardiac surgery. *Exclusion Criteria*: Preoperative diagnosis of Down's syndrome plus emergency operations)

The primary end points were the release of troponin-I (enzyme-linked immunosorbent assay; Access Immunoassay System; Beckman Instruments Inc, Fullerton, Calif) and 8-isoprostane (enzyme immunoassay [EIA]; Cayman Chemicals, Ann Arbor, Mich) as measurements of myocardial cell damage and oxidative stress. Secondary endpoint were the release of markers of the whole body inflammatory response (complement activation [C3a], ((BD OptEIA™ Human C3a ELISA)) plus interleukin [IL] 6, IL-8, and IL-10 ((enzyme-linked immunosorbent assay; Amersham Biosciences UK, Little Chalfont, United Kingdom)), and stress response (cortisol; Access Immunoassay System, Beckman Coulter). Cerebral injury was assessed by the postoperative release of protein S100 (CanAg S100 EIA; CanAg Diagnostics AB, Goteborg, Sweden) and alpha-glutathione S-transferase (ἀGT) (Biotrin High Sensitivity Alpha GST EIA Assay; Biotrin International, Dublin, Ireland) was used to assess hepatic cell damage.

Continuous outcomes are summarised as an arithmetic mean and standard deviation if normally distributed or as a geometric mean or median and interquartile range if skewed. Categorical data are presented as actual counts and percentages. Skewed measures were log-transformed to achieve normality and the results were back transformed to the original scale. All the markers had skewed distributions and were analysed on the logarithmic scale.

An overall estimate, pooled over all time points, is reported. Effect sizes are reported as mean differences (if normally distributed) or as ratios of geometric means (if skewed) with corresponding 95% confidence intervals (CIs) and *p* values.

4.1.1 The effect of hyperoxic versus normoxic cardiopulmonary bypass for all recruited patients:

Seventy-nine cyanotic patients were randomised to receive either controlled normoxic (70-100 mm Hg) or hyperoxic (150–200 mm Hg) CPB (**Table 2**). Forty-seven patients (59%) were double ventricular and thirty-two (41%) had functional single-ventricular pathology (Table 7).

Forty patients were randomised to the normoxic and thirty-nine to the hyperoxic CPB group. In the normoxic group 16 patients (40%) had functional single-ventricular and 24 (60%) double-ventricular pathology. In the hyperoxic group, 16 patients (41%) were single-ventricular and 23 (59%) double-ventricular (**Table 2**).

All patients with double-ventricular cyanotic pathology underwent curative surgery. From the 32 patients with single-ventricular pathology, 12 (37.5%) had palliative and 20 (62.5%) had final stage surgery.

Overall, the median age was 438 days (interquartile range 169–1441 days) with 62% male. Reasons for surgery included tetralogy of Fallot (39%), single ventricle (41%), transposition of the great arteries and total anomalous pulmonary venous drainage (20%). Preoperative characteristics are shown in **Table 2**. Intraoperative and clinical outcomes are reported in Table 3.

Categorical outcomes (death and renal failure) were not subject to statistical analysis owing to the small number of events (<5).

Table 2 Baseline Characteristics for All Patients

Variable		Normoxic (n=40)	Hyperoxic (n=39)
Age in days (median (IQR))		448 (178 – 1212)	434 (146 – 1580)
Male (%)		26 (65)	23 (59)
Weight in Kg (median (IQR))		9.6 (7.5-13.9)	9.5 (6.6-15.8)
Renal Failure (%)		3 (8)	2 (5)
Pathology	Single Ventricle Pathology (%)	16 (40)	16 (41)
	TOF (%)	15 (37.5)	16 (41)
	TAPVD & TGA (%)	9 (22.5)	7 (18)

TOF= Tetralogy of Fallot; TAPVD= Total Anomalous Pulmonary Venous Defect; TGA=Transposition of the Great Arteries

Table 3 Operative and Post-operative Details for All Patients

Variable		Normoxic (n=40)	Hyperoxic (n=39)	Effect Size	(95% CI)	p-value
Operative Details	**Oxygen Saturation ***	79.9 (7.61)	78.5 (7.22)	1.34‡	(-2.29 - 4.97)	0.46
	CPB Time (min) §	82.9 (41 – 147)	89.7 (43 – 210)	0.93†	(0.77 - 1.11)	0.40
	Cross-clamp time (min) *	52.0 (20.52)	52.3 (24.66)	-0.39‡	(-14.16 - 13.38)	0.96
Post-Operative Details	**Ventilation time (min) §**	21.5 (2 – 191)	19.6 (3 – 72)	1.12†	(0.67 - 1.87)	0.66
	Length of stay in days (median (IQR))	10.5 (6.0 – 14.0)	8.5 (6.5 – 12.5)	0.90	(0.55 - 1.50)	0.67
	Dopamine on Coming off bypass (µ /kg/min) *	6.3 (3.44)	6.8 (4.84)	-0.53‡	(-2.61 - 1.56)	0.62
	Dopamine Peak dose (µ /kg/min) *	11.8 (5.22)	9.6 (5.36)	2.19‡	(-0.58 - 4.96)	0.12
	Dopamine Duration (hours) §	33.5 (6 – 256)	28.6 (0 – 366)	1.19†	(0.73 - 1.96)	0.48

‡) Difference in means (normoxic – hyperoxic)
†) Ratio of geometric means (normoxic:hyperoxic)
*) Values are expressed as mean (SD)
§) Values are expressed as geometric mean with range

Table 4 Descriptive results for length of hospital stay, CPB time and cross clamp time (All Patients)

					Statistic	Std. Error
All Patients	Bypass Time (minutes)	Single Ventricular	Mean		75.26	5.766
			95% Confidence Interval for Mean	Lower Bound	63.48	
				Upper Bound	87.03	
			5% Trimmed Mean		72.55	
			Median		64.00	
			Variance		1030.665	
			Std. Deviation		32.104	
			Minimum		34	
			Maximum		178	
			Range		144	
			Interquartile Range		29	
		Double Ventricular	Mean		104.32	5.186
			95% Confidence Interval for Mean	Lower Bound	93.88	
				Upper Bound	114.76	
			5% Trimmed Mean		102.16	
			Median		106.00	
			Variance		1263.961	
			Std. Deviation		35.552	
			Minimum		50	
			Maximum		210	
			Range		160	
			Interquartile Range		53	
	Cross-Clamp Time (minutes)	Single Ventricular	Mean		8.81	3.275
			95% Confidence Interval for Mean	Lower Bound	2.12	
				Upper Bound	15.50	
			5% Trimmed Mean		6.50	
			Median		.00	
			Variance		332.561	
			Std. Deviation		18.236	
			Minimum		0	
			Maximum		63	
			Range		63	
			Interquartile Range		7	
		Double Ventricular	Mean		59.45	3.319
			95% Confidence Interval for Mean	Lower Bound	52.77	
				Upper Bound	66.13	
			5% Trimmed Mean		59.05	
			Median		59.00	
			Variance		517.687	
			Std. Deviation		22.753	
			Minimum		12	
			Maximum		120	
			Range		108	
			Interquartile Range		35	
	Length of Stay (days)	Single Ventricular	Mean		16.90	2.833
			95% Confidence Interval for Mean	Lower Bound	11.12	
				Upper Bound	22.69	
			5% Trimmed Mean		14.77	
			Median		11.00	
			Variance		248.824	
			Std. Deviation		15.774	
			Minimum		4	
			Maximum		86	
			Range		82	
			Interquartile Range		15	
		Double Ventricular	Mean		9.55	1.159
			95% Confidence Interval for Mean	Lower Bound	7.22	
				Upper Bound	11.89	
			5% Trimmed Mean		8.36	
			Median		7.00	
			Variance		63.122	
			Std. Deviation		7.945	
			Minimum		4	
			Maximum		55	
			Range		51	
			Interquartile Range		4	

Table 5 Descriptive results for length of hospital stay, CPB time and cross clamp time (Normoxic group)

					Statistic	Std. Error
Normoxic Group	Bypass Time	Single Ventricular	Mean		75.07	10.229
			95% Confidence Interval for Mean	Lower Bound	53.13	
				Upper Bound	97.01	
			5% Trimmed Mean		71.63	
			Median		60.00	
			Variance		1569.638	
			Std. Deviation		39.619	
			Minimum		34	
			Maximum		178	
			Range		144	
			Interquartile Range		42	
		Double Ventricular	Mean		96.58	5.699
			95% Confidence Interval for Mean	Lower Bound	84.79	
				Upper Bound	108.37	
			5% Trimmed Mean		96.31	
			Median		95.50	
			Variance		779.471	
			Std. Deviation		27.919	
			Minimum		50	
			Maximum		147	
			Range		97	
			Interquartile Range		52	
	Cross-Clamp Time	Single Ventricular	Mean		11.60	5.673
			95% Confidence Interval for Mean	Lower Bound	-.57	
				Upper Bound	23.77	
			5% Trimmed Mean		9.39	
			Median		0.00	
			Variance		482.829	
			Std. Deviation		21.973	
			Minimum		0	
			Maximum		63	
			Range		63	
			Interquartile Range		16	
		Double Ventricular	Mean		54.71	4.677
			95% Confidence Interval for Mean	Lower Bound	45.03	
				Upper Bound	64.38	
			5% Trimmed Mean		54.79	
			Median		55.00	
			Variance		525.085	
			Std. Deviation		22.915	
			Minimum		12	
			Maximum		95	
			Range		83	
			Interquartile Range		41	
	Length of Stay (days)	Single Ventricular	Mean		13.07	2.168
			95% Confidence Interval for Mean	Lower Bound	8.42	
				Upper Bound	17.72	
			5% Trimmed Mean		12.52	
			Median		11.00	
			Variance		70.495	
			Std. Deviation		8.396	
			Minimum		4	
			Maximum		32	
			Range		28	
			Interquartile Range		11	
		Double Ventricular	Mean		10.67	2.129
			95% Confidence Interval for Mean	Lower Bound	6.26	
				Upper Bound	15.07	
			5% Trimmed Mean		8.86	
			Median		7.50	
			Variance		108.754	
			Std. Deviation		10.429	
			Minimum		4	
			Maximum		55	
			Range		51	
			Interquartile Range		7	

Table 6 Descriptive results for length of hospital stay, CPB time and cross clamp time (Hyperoxic group)

					Statistic	Std. Error
Hyperoxic Group	Bypass Time (minutes)	Single Ventricular	Mean		75.44	6.105
			95% Confidence Interval for Mean	Lower Bound	62.43	
				Upper Bound	88.45	
			5% Trimmed Mean		73.88	
			Median		66.50	
			Variance		596.263	
			Std. Deviation		24.418	
			Minimum		43	
			Maximum		136	
			Range		93	
			Interquartile Range		34	
		Double Ventricular	Mean		112.39	8.583
			95% Confidence Interval for Mean	Lower Bound	94.59	
				Upper Bound	130.19	
			5% Trimmed Mean		110.05	
			Median		110.00	
			Variance		1694.522	
			Std. Deviation		41.165	
			Minimum		60	
			Maximum		210	
			Range		150	
			Interquartile Range		67	
	Cross-Clamp Time (minutes)	Single Ventricular	Mean		6.19	3.530
			95% Confidence Interval for Mean	Lower Bound	-1.34	
				Upper Bound	13.71	
			5% Trimmed Mean		3.99	
			Median		0.00	
			Variance		199.363	
			Std. Deviation		14.120	
			Minimum		0	
			Maximum		52	
			Range		52	
			Interquartile Range		5	
		Double Ventricular	Mean		62.04	5.362
			95% Confidence Interval for Mean	Lower Bound	50.92	
				Upper Bound	73.16	
			5% Trimmed Mean		62.23	
			Median		63.00	
			Variance		661.225	
			Std. Deviation		25.714	
			Minimum		0	
			Maximum		120	
			Range		120	
			Interquartile Range		30	
	Length of Stay (days)	Single Ventricular	Mean		20.50	5.021
			95% Confidence Interval for Mean	Lower Bound	9.80	
				Upper Bound	31.20	
			5% Trimmed Mean		17.72	
			Median		12.50	
			Variance		403.333	
			Std. Deviation		20.083	
			Minimum		5	
			Maximum		86	
			Range		81	
			Interquartile Range		21	
		Double Ventricular	Mean		8.39	.821
			95% Confidence Interval for Mean	Lower Bound	6.69	
				Upper Bound	10.09	
			5% Trimmed Mean		8.00	
			Median		7.00	
			Variance		15.522	
			Std. Deviation		3.940	
			Minimum		5	
			Maximum		19	
			Range		14	
			Interquartile Range		4	

Table 7 Preoperative and Operative Descriptive Results for Single Ventricular and Double Ventricular Patients

Variables	Single-Ventricular (n=32)	Double-Ventricular (n=47)
Mean age at surgery (days)	1771.5	333.64
Mean preoperative O_2 Saturation	79.41	79.74
Mean CPB time	75.26	104.32
Number of patients who had their surgery with cross clamp	8 (25%)	47 (100%)
Mean postoperative intubation time	16.52	49.15
Mean aortic cross clamp time	8.81 (34.1)*	59.45

* Figure presented in the bracket applies to only single ventricular patients who had their surgery with aortic cross clamp

Table 8 Biochemical Markers for All Patients

Variable	Time	Geometric mean*		Ratio	(95% CI)	P-value
		Normoxic	Hyperoxic			
Troponin I		N=37	N=38			
	Pre-Op	0.019	0.017			
	10 min on CPB	0.25	0.31	0.81		
	30 min on CPB	0.90	0.96	0.94		
	10 min Off CPB	4.75	8.34	0.57		
	4 Hrs post CPB	5.67	8.5	0.67		
	24 Hrs post CPB	4.19	5.8	0.72		
	Test for interaction between treatment and time					**0.471**
	Treatment effect, pooled over all time point			**0.74**	**(0.71-0.77)**	**<0.01**
8-Iso-Prostane		N=39	N=37			
	Pre-Op	2.8	2.3			
	10 min on CPB	5.75	8.71	0.66		
	30 min on CPB	7.04	9.23	0.76		
	10 min Off CPB	6.49	8.91	0.72		
	4 Hrs post CPB	3.75	4.87	0.76		
	24 Hrs post CPB	1.81	3.54	0.51		
	Test for common ratio					**0.71**
	Treatment effect, pooled over all time points			**0.68**	**(0.59-0.78)**	**<0.01**
IL-6		N=39	N=39			
	Pre-op	0.7	0.5			
	10 min on CPB	0.508	0.75	0.68		
	30 min on CPB	0.545	0.67	0.81		
	10 min off CPB	5.57	5.95	0.93		
	4 Hrs post CPB	33.9	45.1	0.76		
	24 Hrs Post CPB	50.2	47.2	1.07		
	Test for interaction between treatment and time					**0.84**
	Treatment effect, pooled over all time points			**0.85**	**(0.81-0.89)**	**<0.01**
IL-8		N=39	N=39			
	Pre-op	5.2	4.5			
	10 min on CPB	6.96	8.03	0.87		
	30 min on CPB	7.83	9.29	0.85		
	10 min off CPB	18.05	21.11	0-85		
	4 Hrs post CPB	38.24	36.95	1.02		
	24 Hrs Post CPB	24.69	27.33	0.91		
	Test for interaction between treatment and time					**0.733**
	Treatment effect, pooled over all time points			**0.89**	**(0.83-0.95)**	**<0.01**
IL-10		N=39	N=39			
	Pre-op	9.3	11.2			
	10 min on CPB	14.01	19.28	0.72		
	30 min on CPB	21.73	28.35	0.78		
	10 min off CPB	132.11	227.57	0.58		
	4 Hrs post CPB	98.26	118.31	0.83		
	24 Hrs Post CPB	30.4	22.21	1.35		
	Test for interaction between treatment and time					**0.347**
	Treatment effect, pooled over all time points			**0.81**	**(0.63-1.07)**	**0.13**
C3 Alpha		N=39	N=39			
	Pre-op	1485.6	1410.2			
	10 min on CPB	1293.23	1313.41	1		
	30 min on CPB	1345.48	1542.85	0.98		
	10 min off CPB	1574.55	1818.88	0.98		
	4 Hrs post CPB	1266.82	1387.72	1		
	24 Hrs Post CPB	1006.4	1049.36	1		
	Test for interaction between treatment and time					**0.355**
	Treatment effect, pooled over all time points			**1**	**(0.98-1.01)**	**0.28**
Cortisol		N=36	N=39			
	Pre-op	493.66	416.05			
	10 min on CPB	281.06	296.27	1		
	30 min on CPB	276.73	305.61	0.98		
	10 min off CPB	242.06	313.99	0.98		
	4 Hrs post CPB	199.34	301.8	0.95		
	24 Hrs Post CPB	405.04	499.63	0.98		
	Test for interaction between treatment and time					**0.182**
	Treatment effect, pooled over all time points			**0.98**	**(0.95-1.02)**	**0.31**
S100		N=38	N=39			
	Pre-op	226.4	229.5			
	10 min on CPB	343.8	473.21	0.72		
	30 min on CPB	660.26	703.3	0.93		
	10 min off CPB	1161.39	1519.79	0.76		
	4 Hrs post CPB	304.92	311.71	0.98		
	24 Hrs Post CPB	141.92	209.5	0.68		
	Test for interaction between treatment and time					**0.028**
	Treatment effect, pooled over all time points			**0.81**	**(0.76-0.91)**	**<0.01**
Alpha GT		N=38	N=39			
	Pre-op	3183.75	3110.16			
	10 min on CPB	3235.47	4155.51	0.78		
	30 min on CPB	4298.62	4800.91	0.89		
	10 min off CPB	5602.64	7245.76	0.78		
	4 Hrs post CPB	7334.83	8884.85	0.83		
	24 Hrs Post CPB	3678.26	4857.65	0.76		
	Test for interaction between treatment and time					**0.2**
	Treatment effect, pooled over all time points			**0.81**	**(0.76-0.87)**	**<0.01**

4.1.2 The effect of hyperoxic versus normoxic cardiopulmonary bypass on patients with functional single ventricular pathology:

Out of seventy-nine patients who were recruited for this study, thirty-two (41%) had functional single-ventricular pathology (Table 7) of which, 16 patients were randomised to receive normoxic CPB and 16 to hyperoxic (Table 9). The mean CPB time for patients with single-ventricular pathology was 75.26 minutes. Twenty-four patients (75%) had their surgery without aortic cross-clamp and eight patients (25%) had their aorta cross-clamped with a mean duration of 34.1 minutes (minimum 7 and maximum of 63 minutes) (Table 7).

In the normoxic group 5 patients (31%) had palliative and 11 (69%) had final-stage surgery. In the hyperoxic group 7 patients (44%) had palliative and 9 (56%) had final stage surgery.

In single-ventricular group, the median age was 1545 days (interquartile range 820–2028 days) with 62.5% male. Baseline characteristics are summarised in Table 9. Intraoperative and clinical outcomes are reported in Table 10.

Table 9 Baseline Characteristics for Patients with Single Ventricular Pathology

Variable		Normoxic (n=16)	Hyperoxic (n=16)
Age in days (median (IQR))		1476 (820-2028)	1621.5 (664.2473)
Male (%)		10 (62.5)	10 (62.5)
Weight in Kg (median (IQR))		13.9 (10.6-19.6)	16.1 (11.6-19.6)
Death		1 (6.2)	1(6.2)
Renal Failure (%)		3(19)	0 (0)
Pathology	Pulmonary Atresia/Stenosis (%)	11(69)	5(31)
	Double-Inlet AV Connection (%)	2 (13)	7 (44)
	Ebstein's Anomaly (%)	1(6)	2(13)
	Tricuspid Atresia/Stenosis (%)	1(6)	2(13)
	Mitral Stenosis (%)	1(6)	0(0)

Table 10 Operative and Post-operative Details for Patients with Single Ventricular Pathology

Variable		Normoxic (n=16)	Hyperoxic (n=16)	Effect Size	(95% CI)	p-value
Operative Details	**Oxygen Saturation***	81.06 (7.56)	77.75 (7.19)	3.31‡	(-2.01-8.64)	0.21
	CPB Time (min) §	67.23 (34-178)	72.21 (43 –136)	0.93†	(0.77 - 1.11)	0.97
	Cross-clamp time (min) *	11.6 (21.97)	6.19 (14.12)	5.41‡	(-8.06 – 18.89)	0.42
Post-Operative Details	**Ventilation time (min) §**	21.20 (2 – 188)	11.50 (2-33)	1.84†	(0.67 - 1.87)	0.45
	Length of stay in days (median (IQR))	13.07 (6.0-17.0)	20.5 (7.75-28.5)	0.90	(0.55 - 1.50)	0.67
	Dopamine on Coming off bypass (µ /kg/min) *	5.31 (5.35)	6.17 (4.71)	-0.86‡	(-3.90-2.19)	0.57
	Dopamine Peak dose (µ /kg/min) *	9.81 (5.34)	10.33 (6.33)	-0.52‡	(-5.12-4.07)	0.82
	Dopamine Duration (hours) *	40.36 (62.89)	62.63 (62.63)	-22.27‡	(-91.75-47.20)	0.52

‡) Difference in means (normoxic – hyperoxic)
†) Ratio of geometric means (normoxic:hyperoxic)
*) Values are expressed as mean (SD)
§) Values are expressed as geometric mean with range

Table 11 Biochemical Markers for Patients with Single-Ventricular Pathology

Variable	Time	Geometric mean*		Ratio	(95% CI)	P-value
		Normoxic	Hyperoxic			
Troponin I		N=15	N=16			
	Pre-Op	0.01	0.01			
	10 min on CPB	0.15	0.29	0.53		
	30 min on CPB	0.38	0.85	0.44		
	10 min Off CPB	1.12	2.19	0.51		
	4 Hrs post CPB	1.75	2.55	0.69		
	24 Hrs post CPB	1.45	2.21	0.66		
	Test for interaction between treatment and time					**0.465**
	Treatment effect, pooled over all time point			**0.57**	**0.51-0.65**	**<0.01**
8-Iso-Prostane		N=16	N=15			
	Pre-Op	3.93	3.83			
	10 min on CPB	6.13	8.29	0.74		
	30 min on CPB	7.85	9.83	0.80		
	10 min Off CPB	6.93	8.65	0.80		
	4 Hrs post CPB	5.62	7.09	0.79		
	24 Hrs post CPB	2.55	4.69	0.54		
	Test for common ratio					**0.45**
	Treatment effect, pooled over all time points			**0.73**	**0.65-0.91**	**0.002**
IL-6		N=16	N=16			
	Pre-op	0.21	0.23			
	10 min on CPB	1.07	1.37	0.78		
	30 min on CPB	2.17	1.87	1.16		
	10 min off CPB	6.43	13.22	0.49		
	4 Hrs post CPB	29.51	53.27	0.55		
	24 Hrs Post CPB	34.63	35.65	0.97		
	Test for interaction between treatment and time					**0.39**
	Treatment effect, pooled over all time points			**0.79**	**0.46-0.93**	**0.02**
IL-8		N=16	N=16			
	Pre-op	4.32	3.70			
	10 min on CPB	6.36	8.28	0.77		
	30 min on CPB	7.25	8.57	0.85		
	10 min off CPB	13.57	16.30	0.83		
	4 Hrs post CPB	35.60	37.66	0.95		
	24 Hrs Post CPB	15.94	21.69	0.73		
	Test for interaction between treatment and time					**0.9**
	Treatment effect, pooled over all time points			**0.83**	**0.78-0.87**	**<0.01**
IL-10		N=16	N=16			
	Pre-op	9.87	10.01			
	10 min on CPB	19.28	29.38	0.66		
	30 min on CPB	27.77	56.44	0.49		
	10 min off CPB	133.14	298.21	0.45		
	4 Hrs post CPB	107.43	148.13	0.73		
	24 Hrs Post CPB	17.25	16.45	1.05		
	Test for interaction between treatment and time					**0.1**
	Treatment effect, pooled over all time points			**0.67**	**0.58-1.05**	**0.1**
C3 Alpha		N=16	N=16			
	Pre-op	1482.86	1788.74			
	10 min on CPB	1400.23	1680.48	0.83		
	30 min on CPB	1357.63	1972.52	0.69		
	10 min off CPB	1578.53	1890.55	0.83		
	4 Hrs post CPB	1176.13	1544.28	0.76		
	24 Hrs Post CPB	1015.28	1247.92	0.81		
	Test for interaction between treatment and time					**0.52**
	Treatment effect, pooled over all time points			**0.79**	**0.81-0.93**	**<0.01**
Cortisol		N=14	N=16			
	Pre-op	383.58	329.57			
	10 min on CPB	238.49	238.82	1.00		
	30 min on CPB	215.84	231.31	0.93		
	10 min off CPB	211.90	230.65	0.92		
	4 Hrs post CPB	325.47	492.47	0.66		
	24 Hrs Post CPB	664.83	770.54	0.86		
	Test for interaction between treatment and time					**0.53**
	Treatment effect, pooled over all time points			**0.87**	**0.83-0.99**	**0.04**
S100		N=16	N=16			
	Pre-op	162.13	147.32			
	10 min on CPB	314.63	442.82	0.71		
	30 min on CPB	580.59	585.87	0.99		
	10 min off CPB	762.02	966.13	0.79		
	4 Hrs post CPB	242.71	262.35	0.93		
	24 Hrs Post CPB	115.36	167.63	0.69		
	Test for interaction between treatment and time					**0.1**
	Treatment effect, pooled over all time points			**0.82**	**0.66-0.91**	**0.002**
Alpha GT		N=16	N=16			
	Pre-op	3198.85	2656.74			
	10 min on CPB	3222.60	4210.77	0.77		
	30 min on CPB	4362.81	5125.47	0.85		
	10 min off CPB	5338.84	6296.62	0.85		
	4 Hrs post CPB	5935.78	9290.39	0.64		
	24 Hrs Post CPB	3423.56	6604.94	0.52		
	Test for interaction between treatment and time					**0.52**
	Treatment effect, pooled over all time points			**0.72**	**0.60-0.85**	**<0.01**

4.1.3 The effect of hyperoxic versus normoxic cardiopulmonary bypass on patients with double ventricular cyanotic pathology:

Forty-seven from a total of seventy-nine patients (59%) who were recruited for this study had biventricular cyanotic pathology (Table 7). 24 patients were randomised to the normoxic and 23 to the hyperoxic arm of this study (Table 12). Overall, the median age was 222 days (interquartile range 40–438 days) with 61.7% male.

All double-ventricular patients underwent curative surgery with aortic cross-clamp. Mean aortic cross-clamp time was 59.45 minutes with minimum of 12 and maximum of 120 minutes. The mean CPB time for patients with biventricular pathology was 104.32 minutes (Table 7).

Baseline characteristics are summarised in Table 12. Intraoperative and clinical outcomes are reported in Table 13.

Table 12 Baseline Characteristics for Double-Ventricular Cyanotic Patients

Variable		Normoxic (n=24)	Hyperoxic (n=23)
Age in days (median (IQR))		217.5 (29 – 550)	223 (74 – 434)
Male (%)		16 (66.7)	13 (56.5)
Weight in Kg (median (IQR))		7.6 (3.6-9.3)	7.6 (4.7-9.8)
Death		0 (0)	0 (0)
Renal Failure (%)		0(0)	2 (9)
Pathology	TOF (%)	15 (62.5)	16 (70)
	TGA (%)	7 (29)	5 (22)
	TAPVD (%)	2 (8)	2 (9)

TOF= Tetralogy of Fallot; TAPVD= Total Anomalous Pulmonary Venous Defect;
TGA=Transposition of the Great Arteries

Table 13 Operative and Post-Operative Details for Double-Ventricular Cyanotic Patients

Variable		Normoxic (n=24)	Hyperoxic (n=23)	Effect Size	(95% CI)	p-value
Operative Details	Oxygen Saturation *	78.08 (10.11)	81.48 (5.35)	-3.4‡	(-8.18-1.39)	0.16
	CPB Time (min) §	92.62 (50-147)	105.48 (60-210)	0.88†	(0.77 - 1.11)	0.97
	Cross-clamp time (min) *	54.71 (22.91)	62.04 (25.71)	7.33‡	(-36.4 – 4.78)	0.13
Post-Operative Details	Ventilation time (min) §	33.64 (6-191)	38.80 (18-287)	0.87†	(-37.3 – 30.4)	0.84
	Length of stay in days (median (IQR))	7.50 (5.25-11.75)	7.00 (6.00-10.0)	1.07	(-2.40- 6.95)	0.33
	Dopamine on Coming off bypass (µ /kg/min) *	7.28 (3.0)	7.17 (4.5)	0.11‡	(-2.18-2.40)	0.92
	Dopamine Peak dose (µ /kg/min) *	12.39 (4.60)	10.13 (4.29)	2.25‡	(-0.57-5.01)	0.11
	Dopamine Duration (hours) *	53.36 (47.37)	51.63 (58.67)	1.73‡	(-31.77-35.24)	0.92

‡) Difference in means (normoxic – hyperoxic)
†) Ratio of geometric means (normoxic:hyperoxic)
*) Values are expressed as mean (SD)
§) Values are expressed as geometric mean with range

Table 14 Biochemical Markers for Patients with Double-Ventricular Cyanotic Pathology

Variable	Time	Geometric mean*		Ratio	(95% CI)	P-value
		Normoxic	Hyperoxic			
Troponin I		N=22	N=22			
	Pre-Op	0.03	0.02			
	10 min on CPB	0.43	0.40	1.10		
	30 min on CPB	1.88	1.25	1.48		
	10 min Off CPB	19.17	20.66	0.91		
	4 Hrs post CPB	18.75	19.86	0.93		
	24 Hrs post CPB	10.98	10.10	1.10		
	Test for interaction between treatment and time					**0.7**
	Treatment effect, pooled over all time point			**1.1**	**0.93 - 1.29**	**0.31**
8-Iso-Prostane		N=23	N=22			
	Pre-Op	3.04	2.28			
	10 min on CPB	5.05	9.67	0.51		
	30 min on CPB	5.23	9.77	0.54		
	10 min Off CPB	5.29	10.46	0.50		
	4 Hrs post CPB	2.58	3.98	0.65		
	24 Hrs post CPB	1.24	2.80	0.44		
	Test for common ratio					**0.9**
	Treatment effect, pooled over all time points			**0.52**	**0.47-0.6**	**<0.01**
IL-6		N=23	N=23			
	Pre-op	0.29	0.27			
	10 min on CPB	0.35	0.35	0.98		
	30 min on CPB	0.22	0.25	0.87		
	10 min off CPB	6.15	2.25	2.75		
	4 Hrs post CPB	40.14	41.32	0.95		
	24 Hrs Post CPB	65.16	61.27	1.05		
	Test for interaction between treatment and time					**0.46**
	Treatment effect, pooled over all time points			**1.15**	**0.87-1.48**	**0.3**
IL-8		N=23	N=23			
	Pre-op	5.51	5.50			
	10 min on CPB	7.37	7.43	1.00		
	30 min on CPB	8.17	9.76	0.83		
	10 min off CPB	22.33	26.00	0.87		
	4 Hrs post CPB	39.11	34.76	1.12		
	24 Hrs Post CPB	37.06	34.35	1.07		
	Test for interaction between treatment and time					**0.29**
	Treatment effect, pooled over all time points			**0.98**	**0.87 - 1.1**	**0.61**
IL-10		N=23	N=23			
	Pre-op	13.45	12.21			
	10 min on CPB	8.65	16.15	0.54		
	30 min on CPB	15.59	16.16	0.95		
	10 min off CPB	122.35	170.96	0.72		
	4 Hrs post CPB	87.07	113.23	0.78		
	24 Hrs Post CPB	32.99	33.20	1.00		
	Test for interaction between treatment and time					**0.6**
	Treatment effect, pooled over all time points			**0.79**	**0.66-0.95**	**0.01**
C3 Alpha		N=23	N=23			
	Pre-op	1489.06	1243.19			
	10 min on CPB	1046.64	1024.24	1.02		
	30 min on CPB	1187.34	1219.30	0.95		
	10 min off CPB	1391.82	1708.55	0.81		
	4 Hrs post CPB	1277.29	1221.53	1.05		
	24 Hrs Post CPB	925.24	943.06	1.00		
	Test for interaction between treatment and time					**0.62**
	Treatment effect, pooled over all time points			**0.85**	**0.78-1.05**	**0.38**
Cortisol		N=22	N=23			
	Pre-op	582.63	480.62			
	10 min on CPB	306.58	344.30	0.89		
	30 min on CPB	329.73	354.34	0.93		
	10 min off CPB	259.80	314.30	0.81		
	4 Hrs post CPB	129.06	188.94	0.68		
	24 Hrs Post CPB	283.37	297.32	0.95		
	Test for interaction between treatment and time					0.68
	Treatment effect, pooled over all time points			**0.85**	**0.78-0.93**	**<0.01**
S100		N=22	N=23			
	Pre-op	286.02	265.21			
	10 min on CPB	382.20	490.88	0.78		
	30 min on CPB	721.49	807.65	0.89		
	10 min off CPB	1707.54	2293.36	0.74		
	4 Hrs post CPB	370.17	346.07	1.07		
	24 Hrs Post CPB	174.64	242.67	0.71		
	Test for interaction between treatment and time					**0.4**
	Treatment effect, pooled over all time points			**0.83**	**0.74-0.93**	**<0.01**
Alpha GT		N=22	N=23			
	Pre-op	3500.18	3855.77			
	10 min on CPB	3240.35	4037.40	0.79		
	30 min on CPB	4066.82	4446.05	0.91		
	10 min off CPB	5815.27	8080.95	0.71		
	4 Hrs post CPB	8340.08	9091.89	0.92		
	24 Hrs Post CPB	3627.80	3765.82	0.95		
	Test for interaction between treatment and time					**0.4**
	Treatment effect, pooled over all time points			**0.87**	**0.78-0.99**	**<0.01**

4.1.3.1 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Troponin-I in All Groups:

Troponin-I was released in a time-dependent fashion as described [283, 377] By 10 minutes after the cessation of CPB, the release of troponin levels were significantly higher than baseline in both groups (Table 8). The levels continued to rise during the 4 hours after surgery (Figure 21) and remained high (compared with baseline) at 24 hours. Overall, troponin-I levels were significantly higher in the hyperoxic group (ratio [normoxic/hyperoxic] = 0.74, 95% CI 0.71-0.77, $P <$ .01)(Table 8).

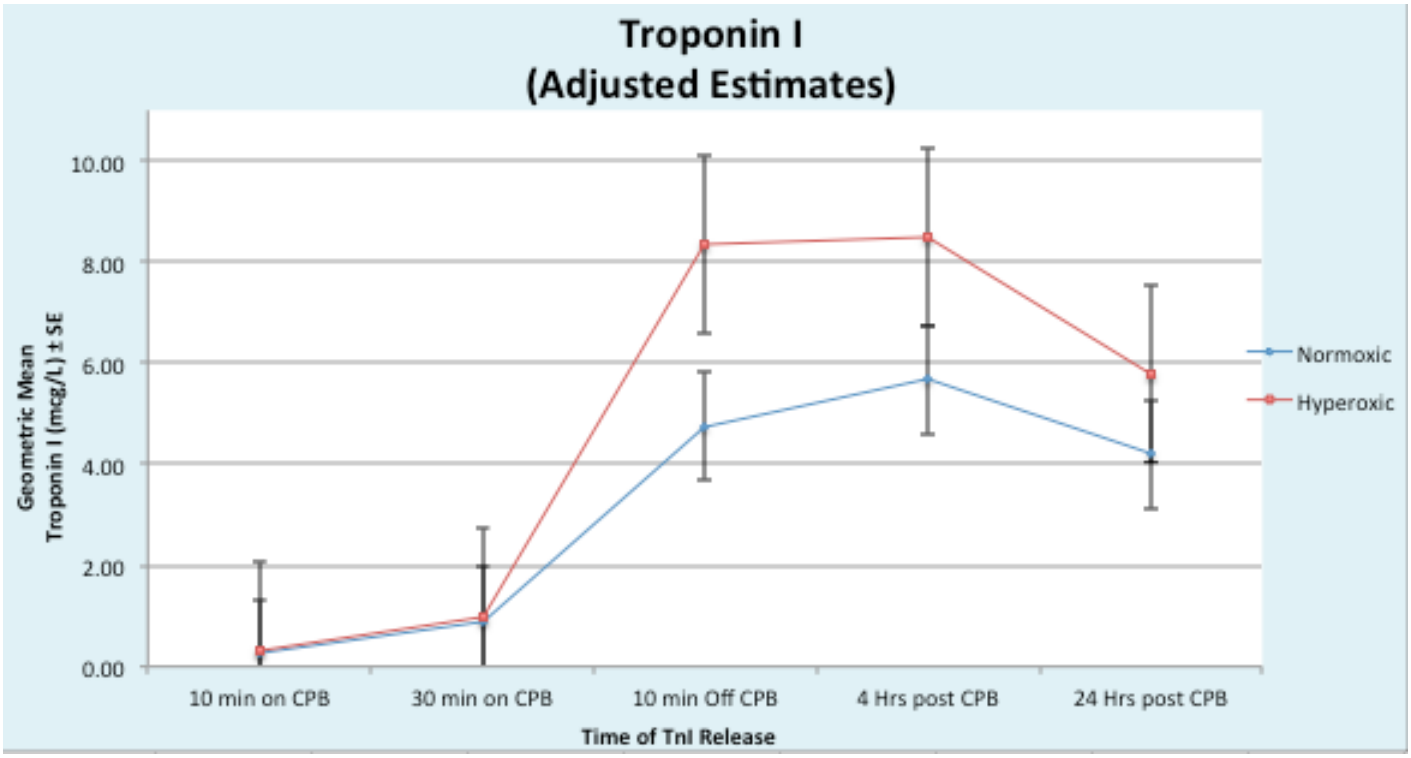

Figure 21 Troponin I release comparison in normoxic and hyperoxic in all patients

4.1.3.2 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass in Release of Troponin-I in Patients With Functional Single-Ventricular Pathology:

By 10 minutes after stopping the CPB, troponin-I levels were significantly higher than baseline in both groups (Table 11). The levels peaked at 4 hours after surgery (Figure 22) and remained high (compared with the baseline) at 24 hours. Overall, troponin-I levels were significantly higher in the hyperoxic group (*ratio [normoxic/hyperoxic] = 0.58, 95% CI 0.51–0.65, P < .01*)(Table 11).

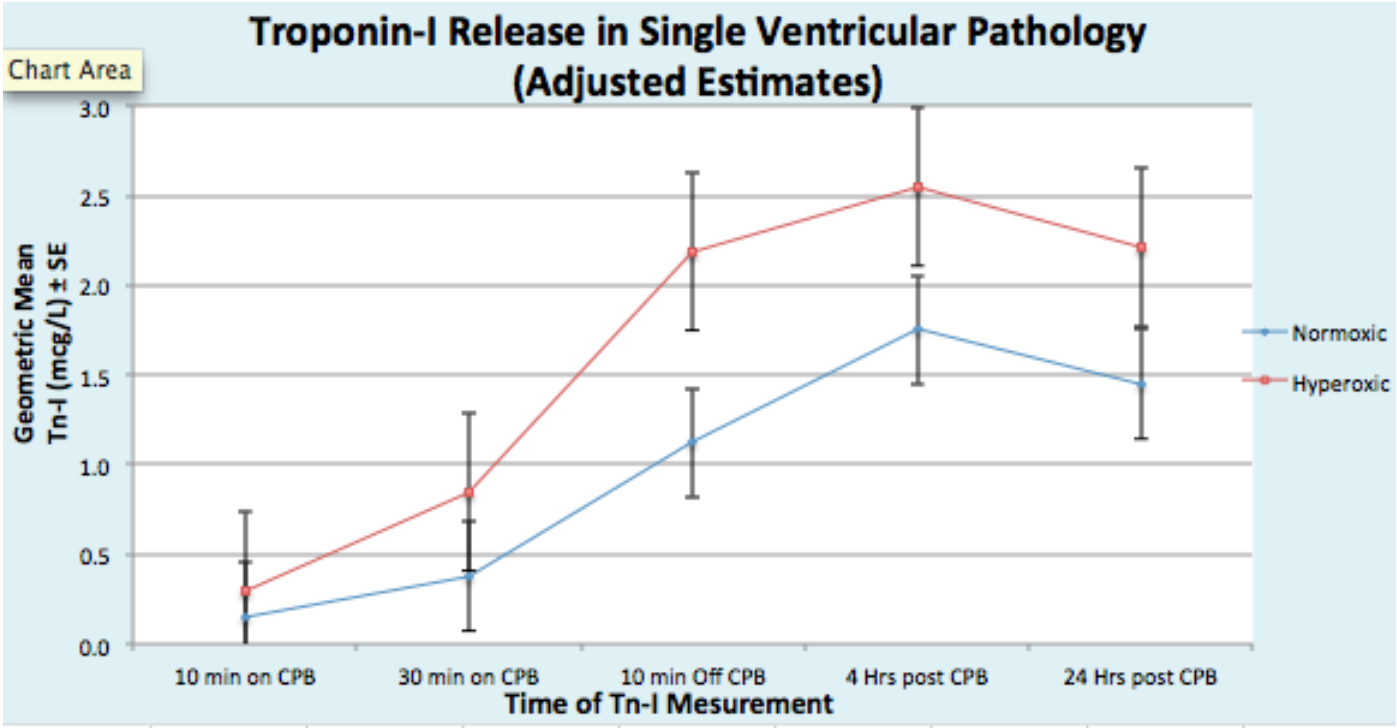

Figure 22 Normoxic vs. hyperoxic Tn-I release in patients with functional single ventricular pathology

4.1.3.3 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Troponin-I in Double-Ventricular Cyanotic Patients:

After 10 minutes of stopping the CPB, Troponin-I levels were significantly higher than baseline in both groups (Table 14). Unlike the single ventricular group that levels continued to raise by 4 hours after surgery, in the biventricular group, the levels peaked at 10 minutes post cessation of CPB and started to decline from this point onwards (Figure 23). There was no evidence to suggest any statistical difference in release of Troponin-I between the normoxic versus hyperoxic groups *(P=0.31)*(Table 14).

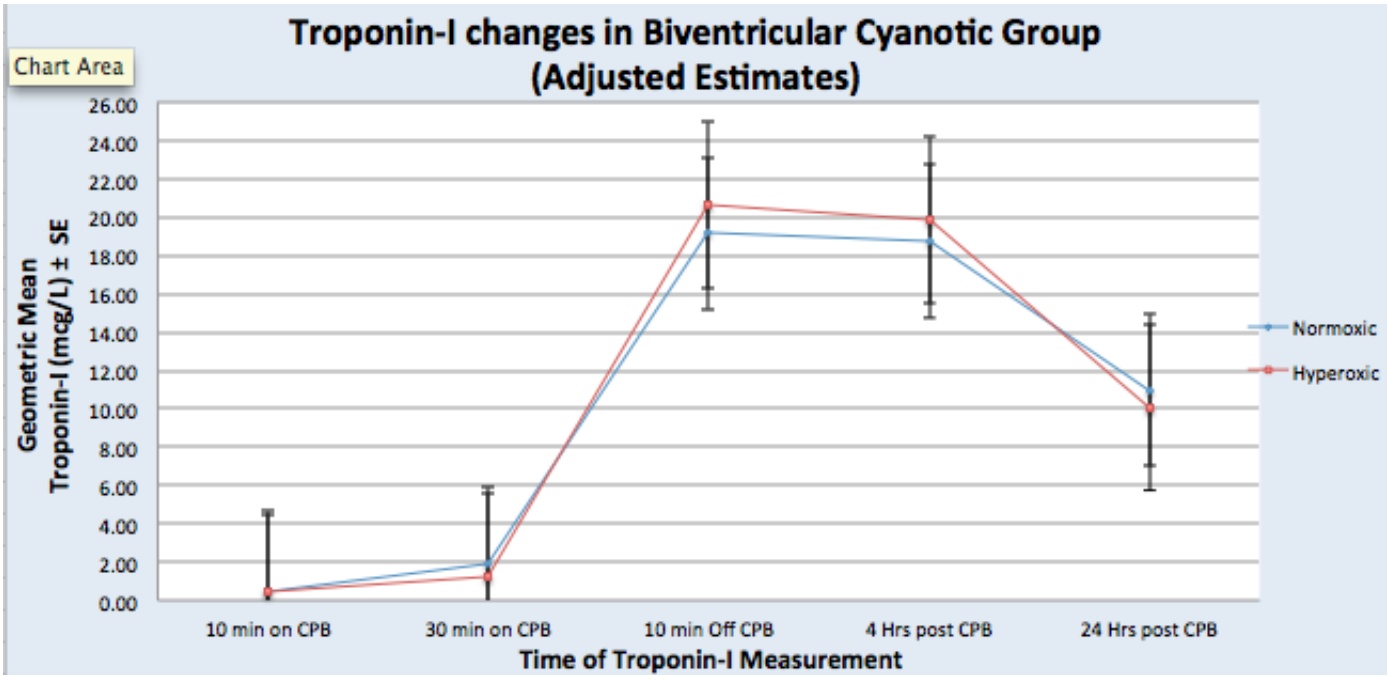

Figure 23 Release of Tn-I in normoxic vs. hyperoxic groups in patients with biventricular cyanotic pathology

4.1.3.4 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of 8-Isoprostane in All Groups:

The release of 8-isoprostane was also time dependent In both groups, levels rose from baseline 10 minutes post initiation of CPB and remained high 10 minutes post coming off CPB, after which time, levels declined (Table 8). The pattern of response was the same in both groups (Figure 24).

Throughout, 8-isoprostane levels were significantly higher in the hyperoxic group (ratio [normoxic/hyperoxic] = 0.68, 95% CI 0.59-0.78, $P < .01$) (Table 8).

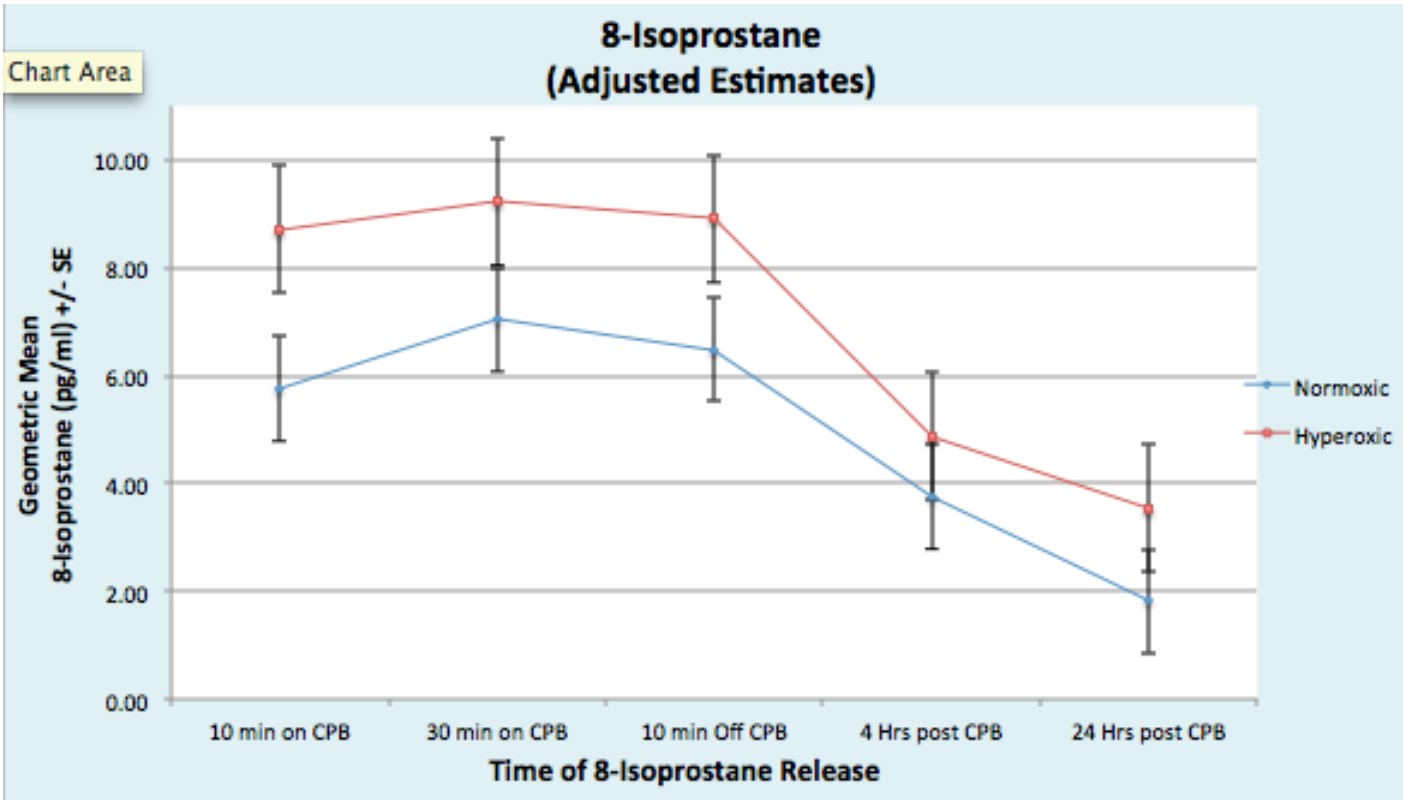

Figure 24 8-Iso-Prostane comparison in normoxic and hyperoxic groups in all patients

4.1.3.5 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of 8-Isoprostane in Patients With Functional Single-Ventricular Pathology:

In both groups, 10 minutes after the start of CPB, levels rose from the baseline (Table 11) and remained high until 30 minutes post initiation of CPB, after which, levels declined with a similar pattern of response in both groups (Figure 25). Throughout, 8-isoprostane levels were significantly higher in the hyperoxic group (*ratio [normoxic/hyperoxic] = 0.76, 95% CI 0.65-0.91, P = .002*) (Table 11).

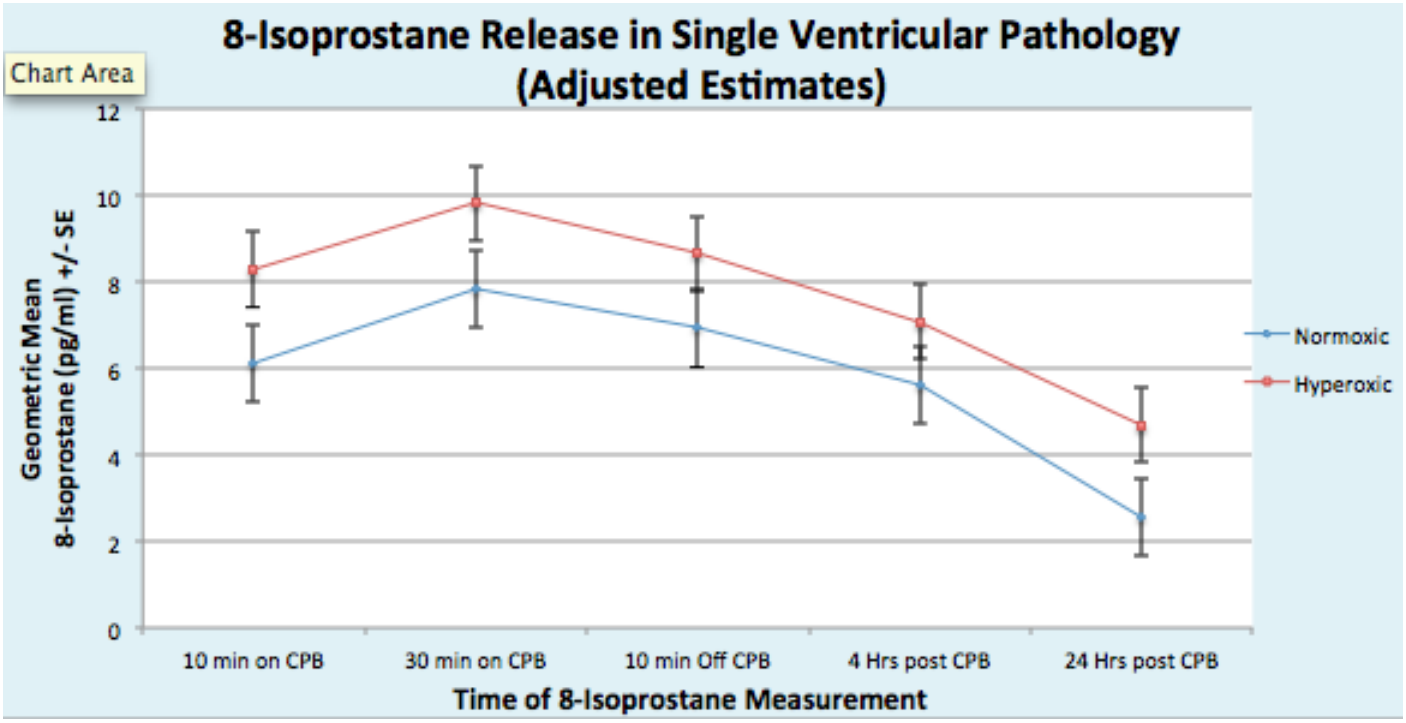

Figure 25 8-Isoprostane release in normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.6 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of 8-Isoprostane in Double-Ventricular Cyanotic Patients:

In both groups, by 10 minutes after the start of CPB, levels rose from the baseline and remained high until 10 minutes after coming off CPB, after which levels declined (Table 14). The response was similar in both groups (Figure 26). The pattern of change in the 8-Isoprostane serum levels was similar between patients with biventricular cyanotic pathology and the general study population. The overall 8-isoprostane levels were significantly higher in the hyperoxic group (ratio [normoxic/hyperoxic] = 0.52, 95% CI 0.47-0.60, P < .01)(Table 14).

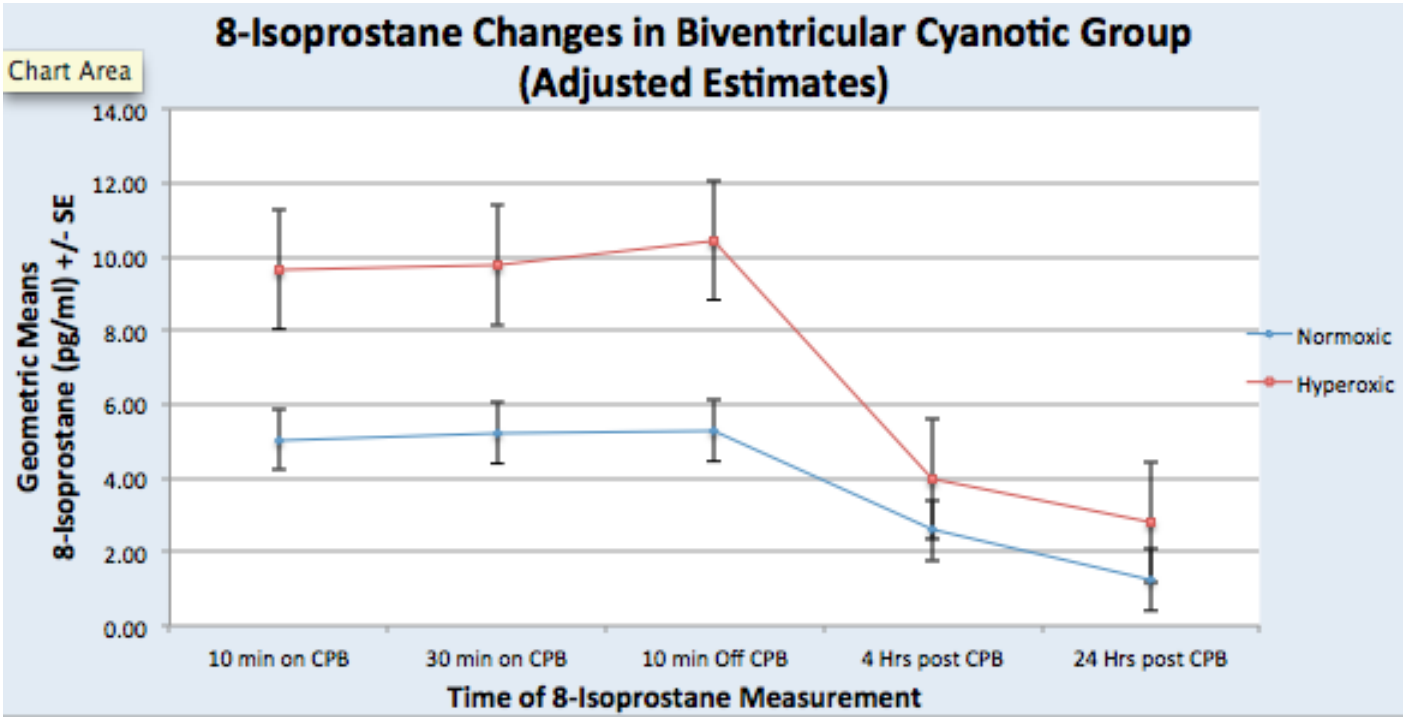

Figure 26 Comparison of 8-Isoprostane levels between normoxic and hyperoxic groups in patients with biventricular cyanotic pathology

4.1.3.7 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Protein S100 in All Groups:

The protein S100 changes were similar in both groups. There was a rise 10 minutes after initiation of CPB (Table 8) and it peaked at 10 minutes after coming off CPB (Figure 27 and Table 8). There was a significant difference in the release of protein S100 between the normoxic versus hyperoxic groups (ratio [normoxic/hyperoxic] = 0.81, 95% CI 0.71–0.91, $P < .01$)(Table 8).

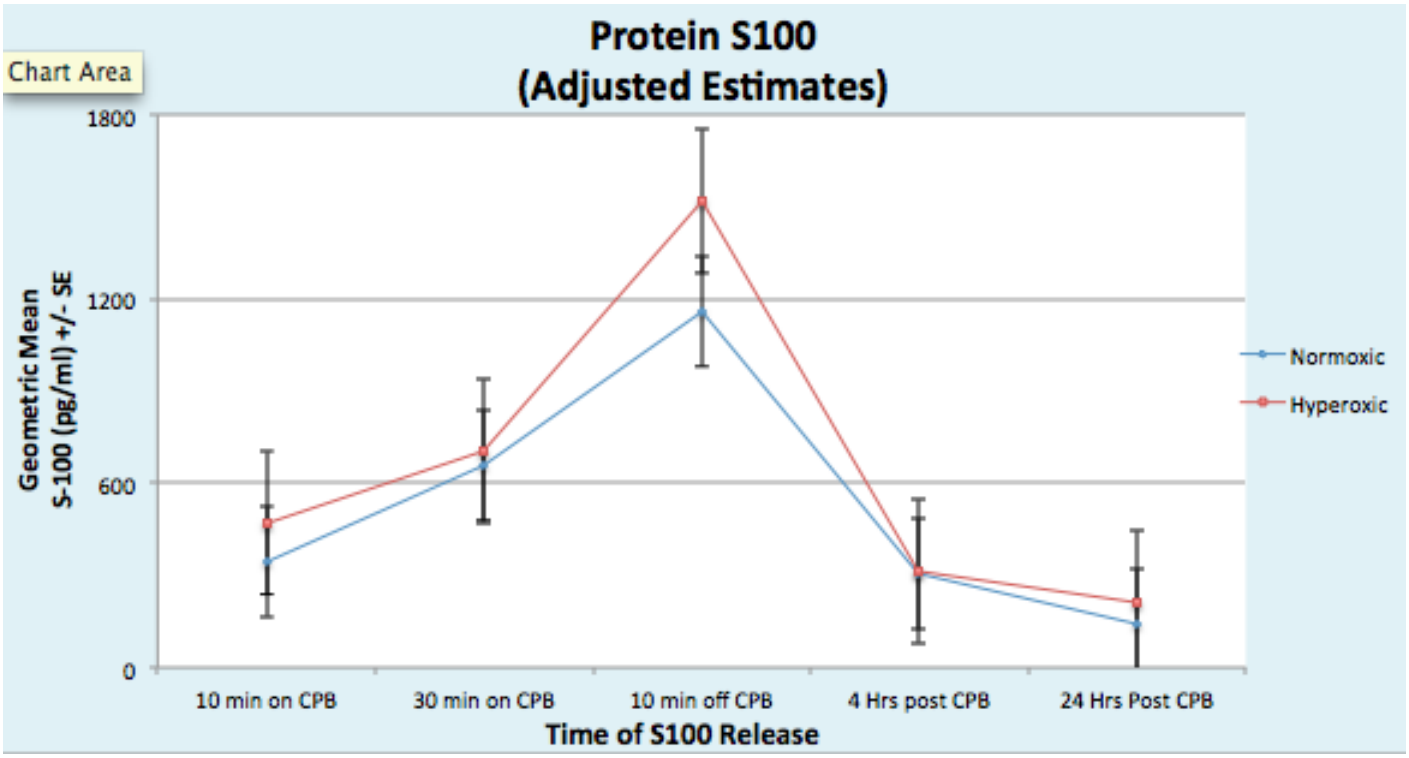

Figure 27 S100 release in Normoxic and Hyperoxic groups in all patients

4.1.3.8 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Protein S100 in Patients With Functional Single-Ventricular Pathology:

The protein S100 changes were similar in both groups (Figure 28 and Table 11). There was a rise 10 minutes after initiation of CPB and it peaked at 10 minutes after coming off CPB (Table 11). There was a significant difference in release of protein S100 between the normoxic and hyperoxic groups (*ratio [normoxic/hyperoxic] = 0.78, 95% CI 0.66–0.91, P = .002*)(Table 11).

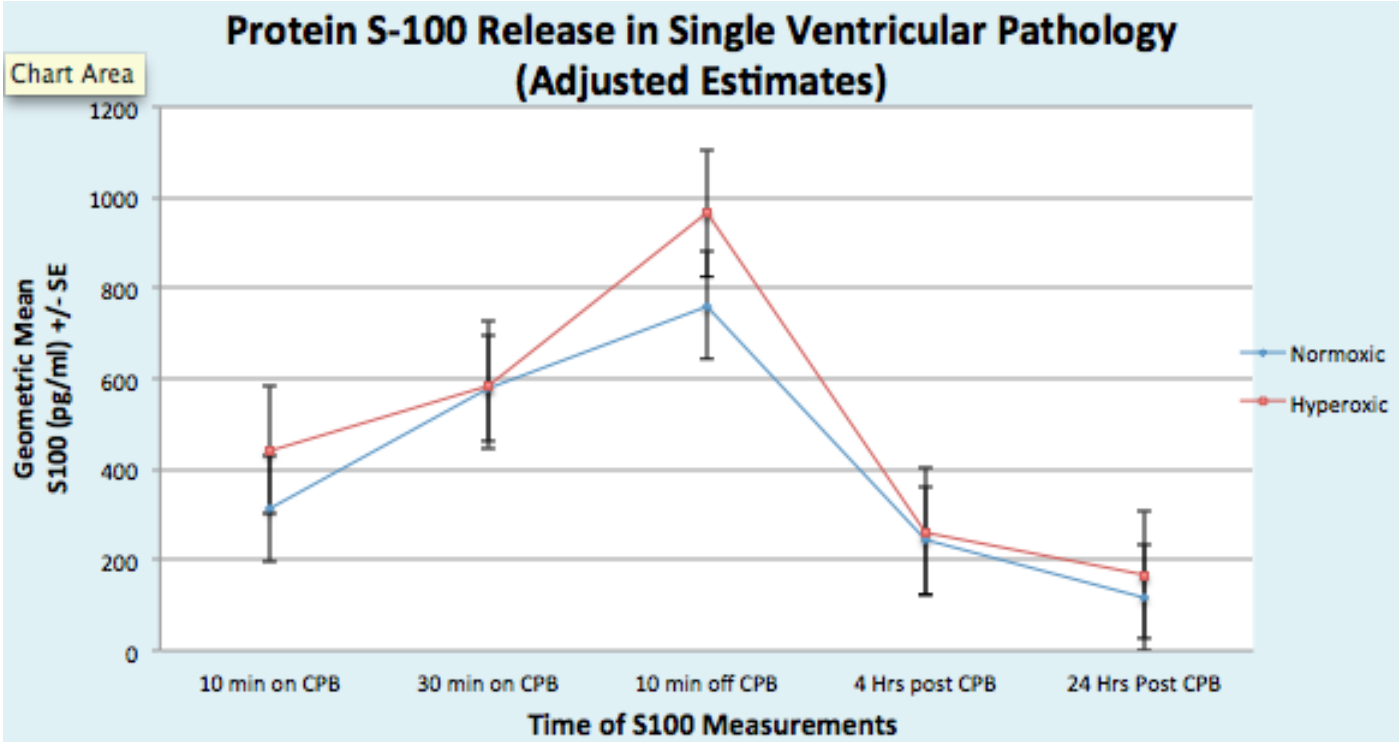

Figure 28 Protein S100 comparison between normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.9 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Protein S100 in Double-Ventricular Cyanotic Patients:

Changes to the levels of serum protein S100 were similar in both normoxic and hyperoxic groups (Figure 29 and Table 14). Compared to the baseline, there was a rise in protein S100, ten minutes after initiation of CPB (Table 14) and it peaked at 10 minutes after coming off CPB. There was a significant difference in Protein S100 levels between the two groups. Patients with biventricular cyanotic pathology that were randomised to the normoxic arm had an overall lower protein S100 levels compared to the hyperoxic group *(ratio [normoxic/hyperoxic] = 0.83, 95% CI 0.74–0.93, P < 0.01)*(Table 14)

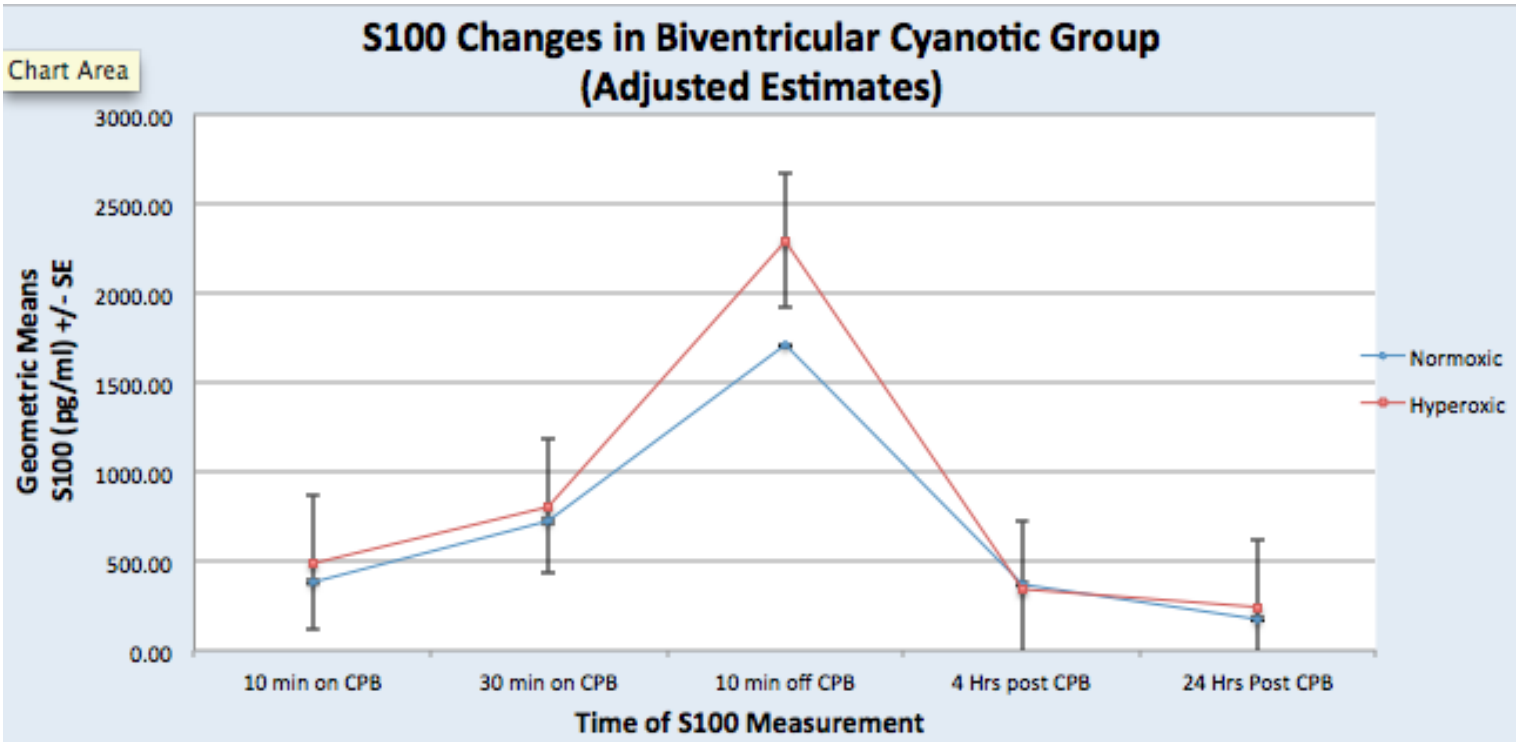

Figure 29 Comparison of serum Protein S100 levels between normoxic vs. hyperoxic groups in patients with double ventricular cyanotic pathology

4.1.3.10 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Alpha GT in All Groups:

There was rise in the άGT in both normoxic and hyperoxic groups compared to the baseline (Table 8). The pattern of changes was similar in both groups and it peaked at 4 hours post termination of CPB (Figure 30 and Table 8). The mean άGT was significantly lower in the normoxic group compared to the hyperoxic group (ratio [normoxic/hyperoxic] = 0.81, 95% CI 0.76–0.87, $P < .01$)(Table 8).

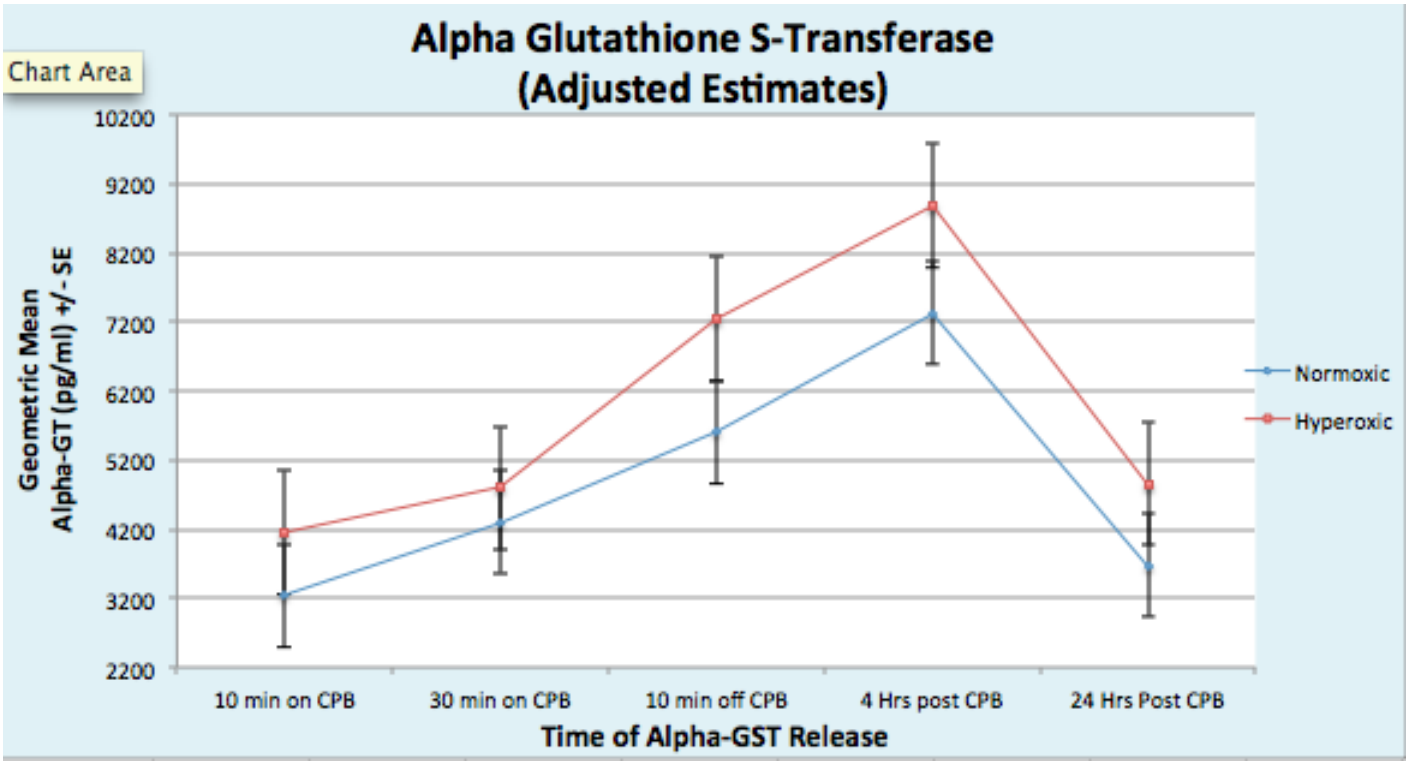

Figure 30 Alpha GT release comparison in normoxic and hyperoxic groups in all patients

4.1.3.11 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Alpha GT in Patients With Functional Single-Ventricular Pathology:

There was rise in the άGT in both normoxic and hyperoxic groups compared to the baseline (Table 11). The pattern of change was similar in both groups and άGT peaked at 4 hours post termination of CPB (Figure 31 and Table 11). The overall άGT was significantly lower in the normoxic group comparing to the hyperoxic (*ratio [normoxic/hyperoxic] = 0.71, 95% CI 0.60–0.85, P < .01*)(Table 11).

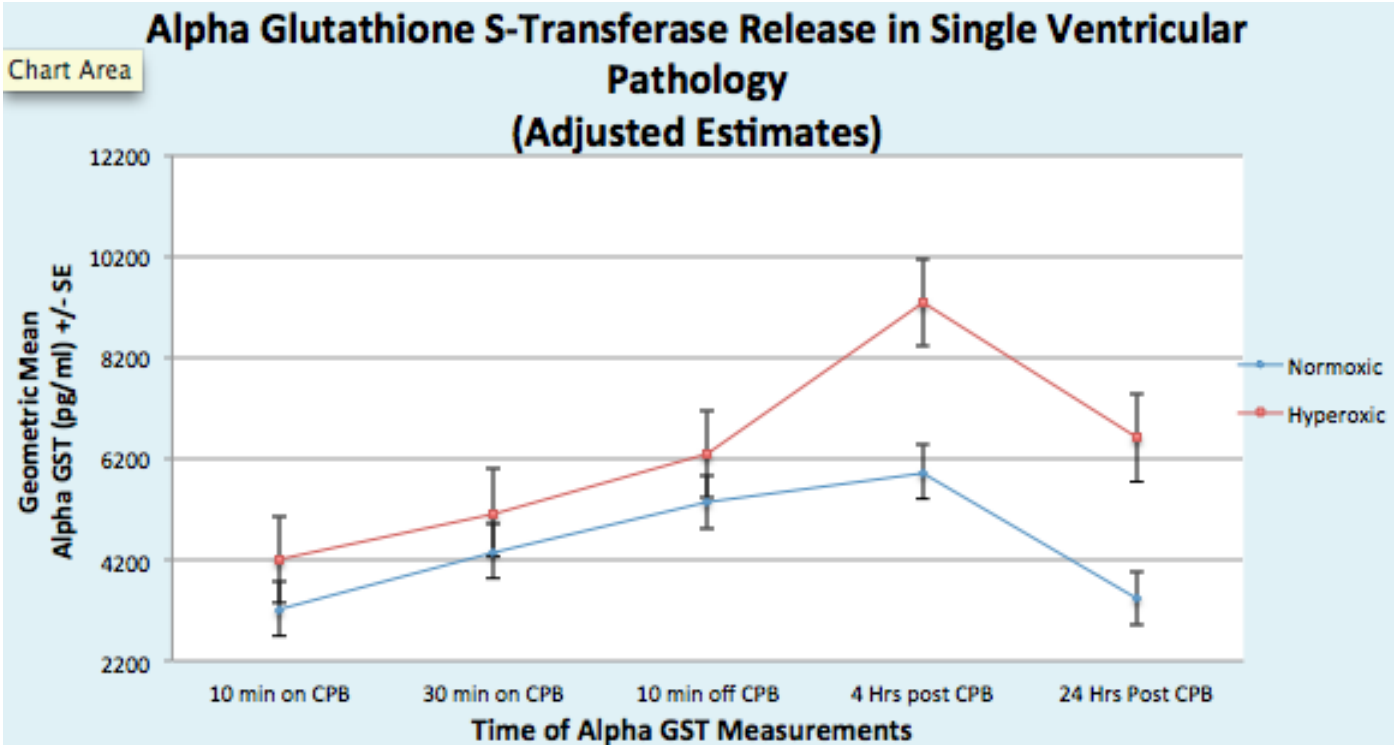

Figure 31 Comparison of aGT levels between normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.12 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Alpha GT in Double-Ventricular Cyanotic Patients:

Patients with biventricular cyanotic pathology, in both the normoxic and hyperoxic groups, had a rise in their plasma άGT levels compared to the baseline (Table 14). The pattern of change was similar in both groups and άGT peaked at 4 hours post termination of CPB (Figure 32 and Table 14). The overall άGT levels were significantly lower in the normoxic group in contrast to the hyperoxic *(ratio [normoxic/hyperoxic] = 0.87, 95% CI 0.78–0.99, P < 0.01)*(Table 14).

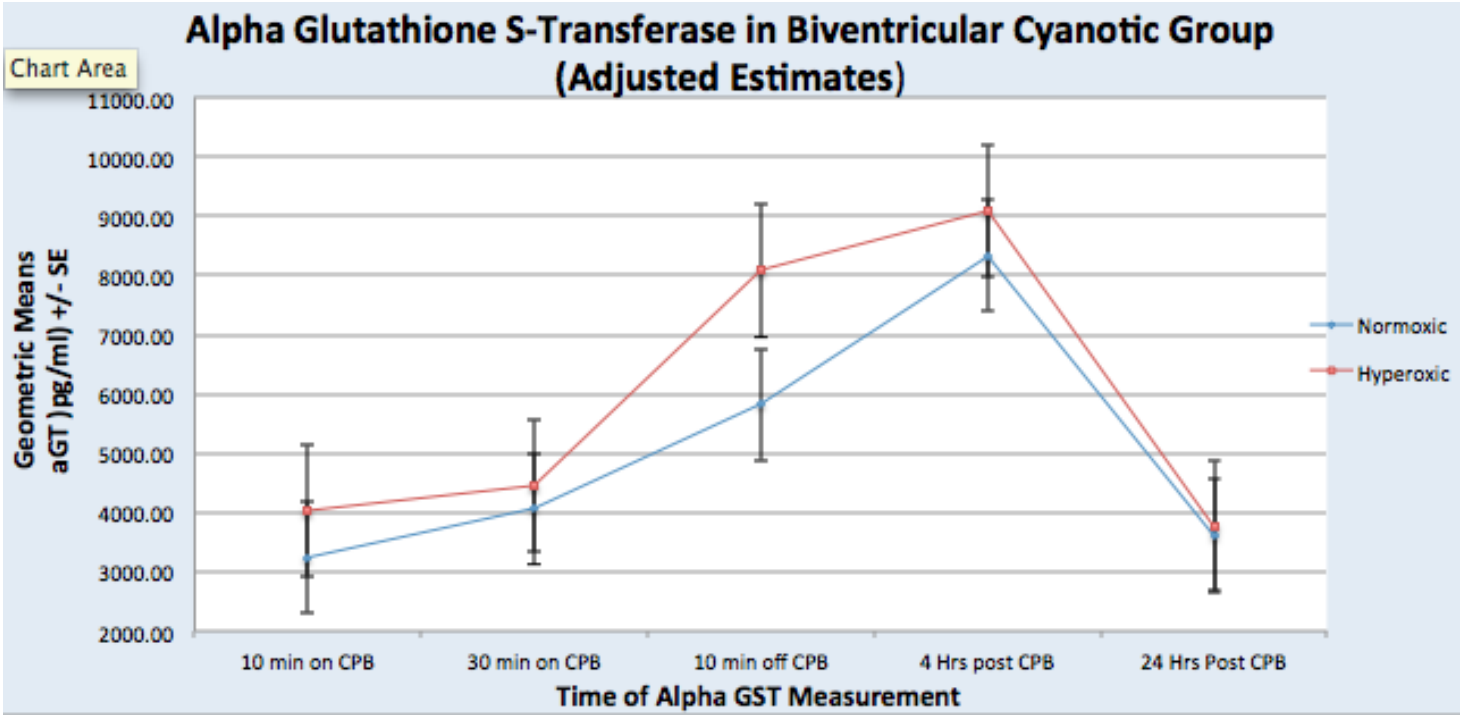

Figure 32 Serum Alpha GT levels in normoxic vs. hyperoxic groups in patients with double-ventricular cyanotic pathology

4.1.3.13 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin 6 (IL-6) in All Groups:

As shown in Figure 33 the pattern of IL-6 release was similar in both hyperoxic and normoxic groups. There was an increase of IL-6 plasma levels in both groups compared to the baseline and it continued to increase 24 hours post cessation of CPB (Table 8). The hyperoxic group had a significantly higher plasma IL-6 levels when compared to the normoxic group (ratio [normoxic/hyperoxic] = 0.85, 95% CI 0.81–0.89, $P < .01$)(Table 8).

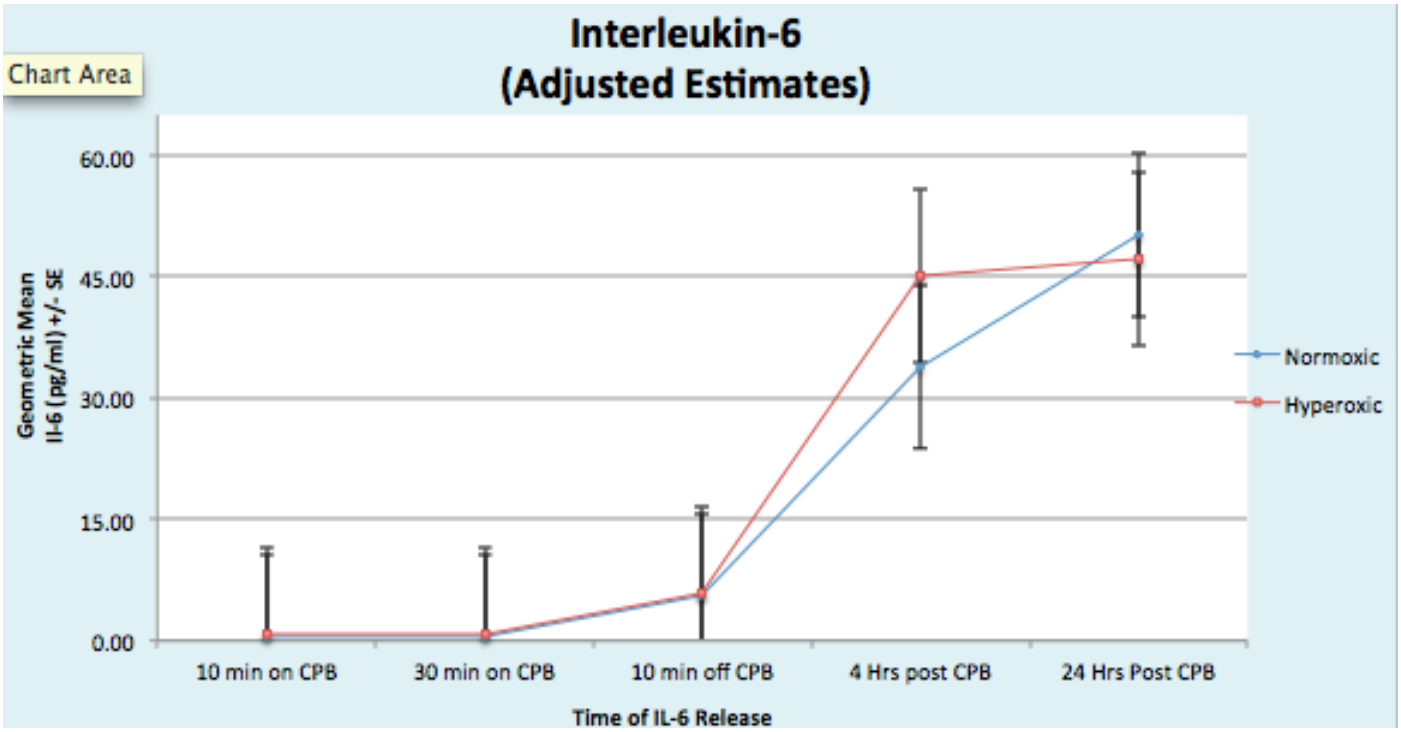

Figure 33 IL-6 comparisons in normoxic and hyperoxic groups in all patients

4.1.3.14 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin 6 (IL-6) in Patients With Functional Single-Ventricular Pathology:

As shown in Table 11, there was an increase of IL-6 plasma levels in both groups compared to the baseline. The IL-6 levels in the normoxic group continued to increase for 24 hours post cessation of CPB. However, in the hyperoxic group there was a decline in the plasma IL-6 levels after 24 hours post termination of CPB (Figure 34 and Table 11). Overall the single-ventricular patients in the hyperoxic group had significantly higher plasma IL-6 levels when compared with normoxic group (*ratio [normoxic/hyperoxic] = 0.66, 95% CI 0.46–0.93, P = .02*) (Table 11).

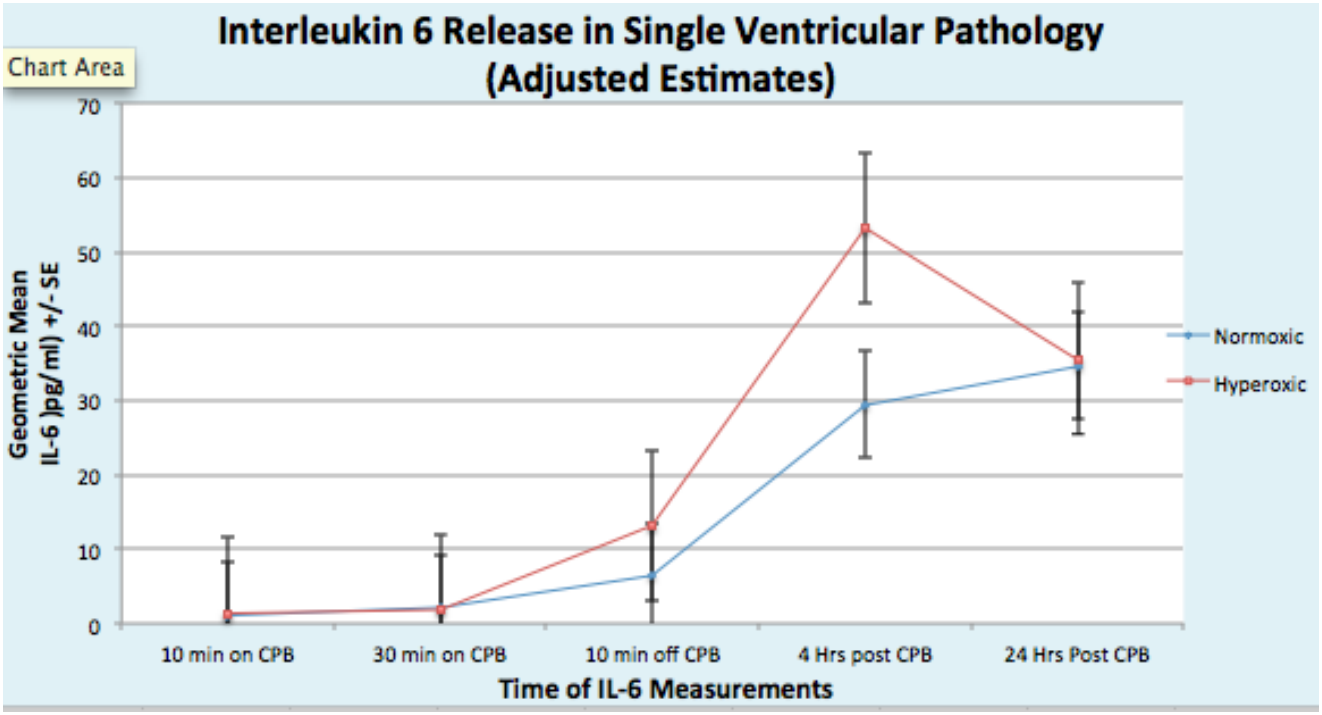

Figure 34 IL-6 levels in normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.15 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin 6 (IL-6) in Double-Ventricular Cyanotic Patients:

As shown in Table 14, there was an increase of IL-6 plasma levels in the normoxic and hyperoxic groups compared to the baseline. The IL-6 levels in both normoxic and hyperoxic groups continued to increase by 24 hours following cessation of CPB (Figure 35). The pattern of IL-6 changes in biventricular group was similar to the overall patients. There was no evidence to suggest any statistically significant difference between the normoxic and hyperoxic groups *(P= 0.3)*(Table 14).

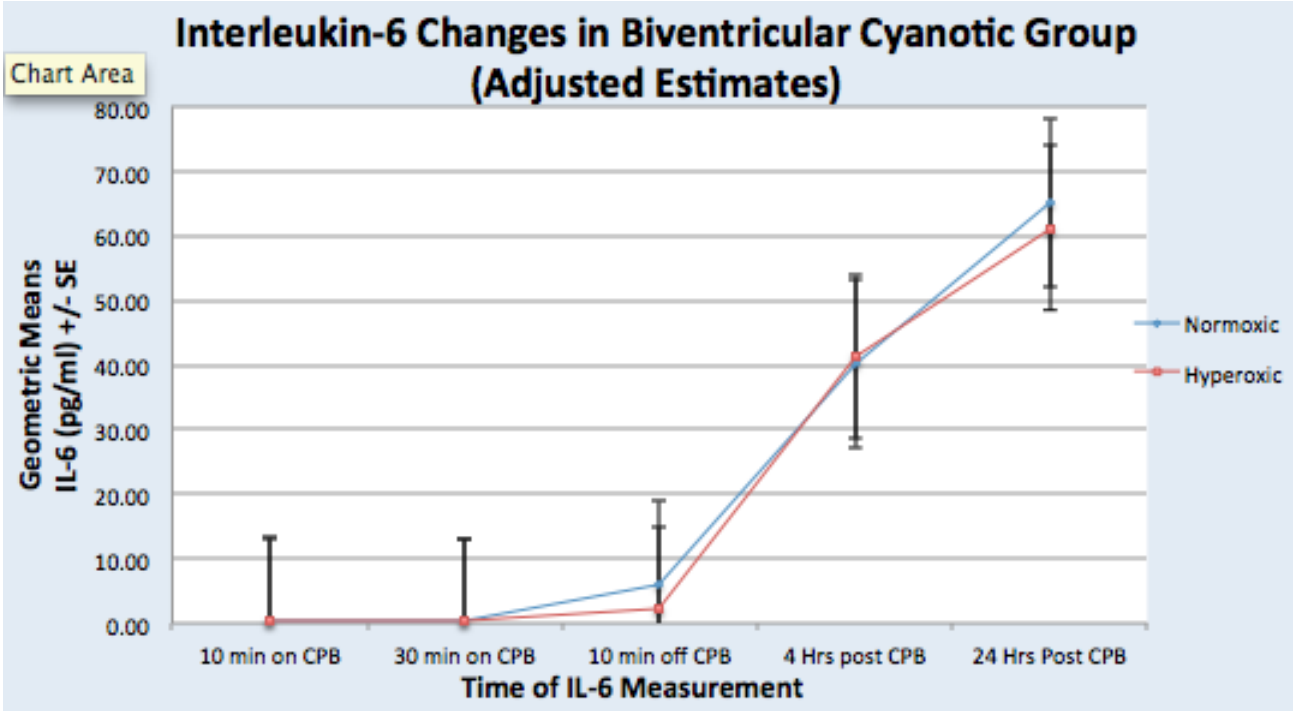

Figure 35 Comparison of interleukin-6 in normoxic vs. hyperoxic groups in patients with double-ventricular cyanotic pathology

4.1.3.16 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin 8 (IL8) in All Groups:

As seen in Table 8, the plasma interleukin 8 showed a rise compared to the baseline in both groups and they both followed a similar pattern. It peaked 4 hours post coming off CPB (Figure 36) Overall, the hyperoxic group had significantly higher plasma IL-8 levels compared to the normoxic group (ratio [normoxic/hyperoxic] = 0.89, 95% CI 0.83–0.95, $P < .01$) (Table 8).

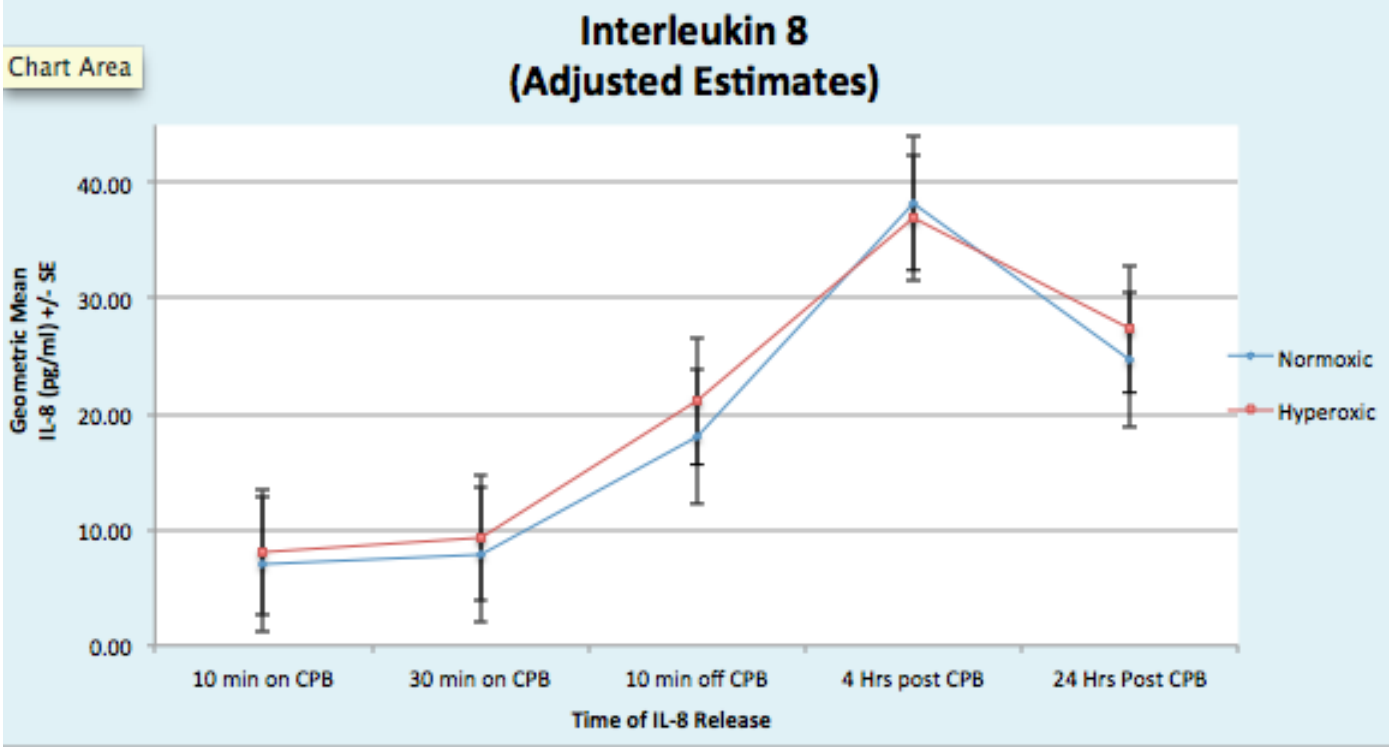

Figure 36 IL-8 release in normoxic and hyperoxic groups in all patients

4.1.3.17 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin 8 (IL-8) in Patients With Functional Single-Ventricular Pathology:

As seen in Table 11, the plasma interleukin-8 showed a rise compared to the baseline in both groups. It peaked at 4 hours after coming off CPB (Figure 37 and Table 11). The pattern of changes were similar in both hyperoxic and normoxic groups. Single-ventricular patients showed a similar pattern to the whole study population regarding changes to the plasma IL-8 levels. The hyperoxic group had significantly higher plasma IL-8 levels compared to the normoxic group (*ratio [normoxic/hyperoxic] = 0.71, 95% CI 0.78–0.87, P < .01*) (Table 11).

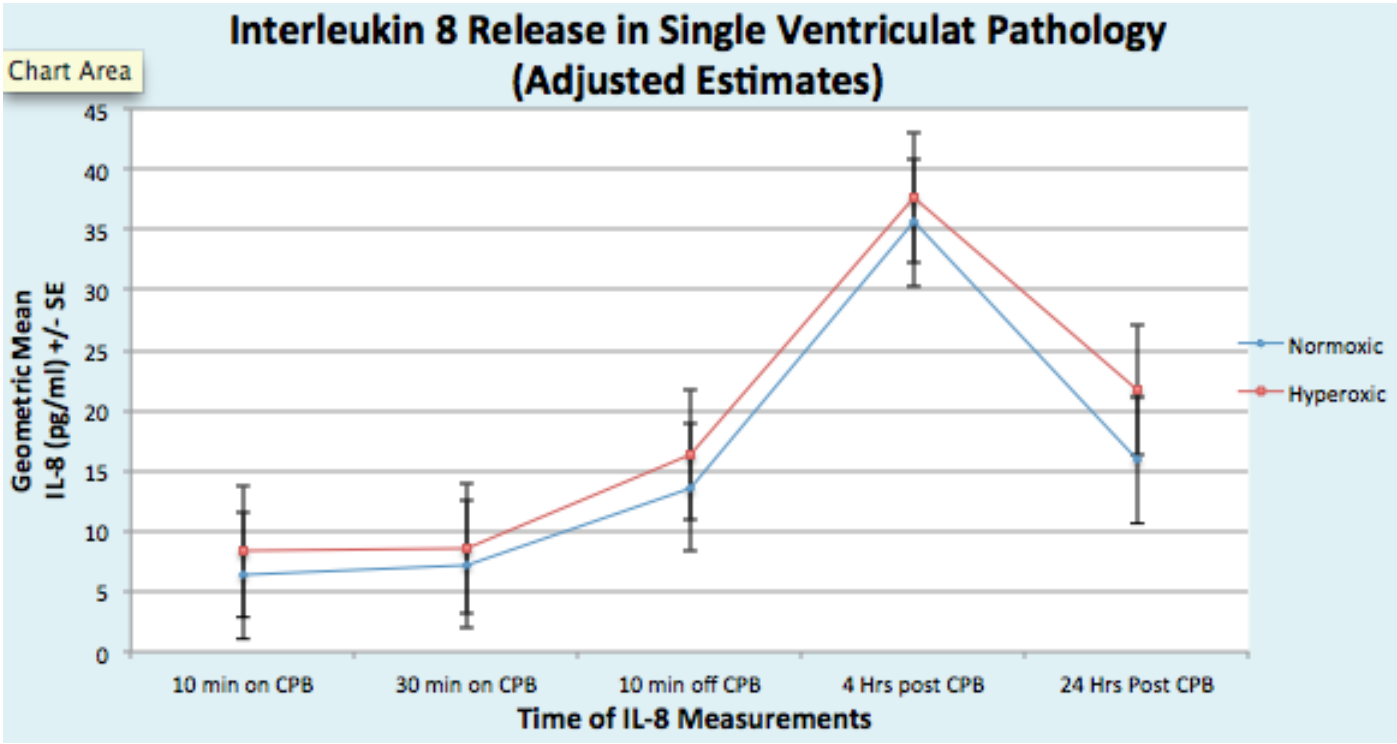

Figure 37 IL-8 levels in normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.18 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin 8 (IL-8) in Double-Ventricular Cyanotic Patients:

Plasma interleukin 8 showed a rise compared to the baseline in both the normoxic and hyperoxic groups (Table 14) and peaked at 4 hours post coming off CPB (Figure 38). There was no difference in IL-8 release between the normoxic versus hyperoxic groups in patients with biventricular cyanotic pathology *(P= 0.61)*(Table 14).

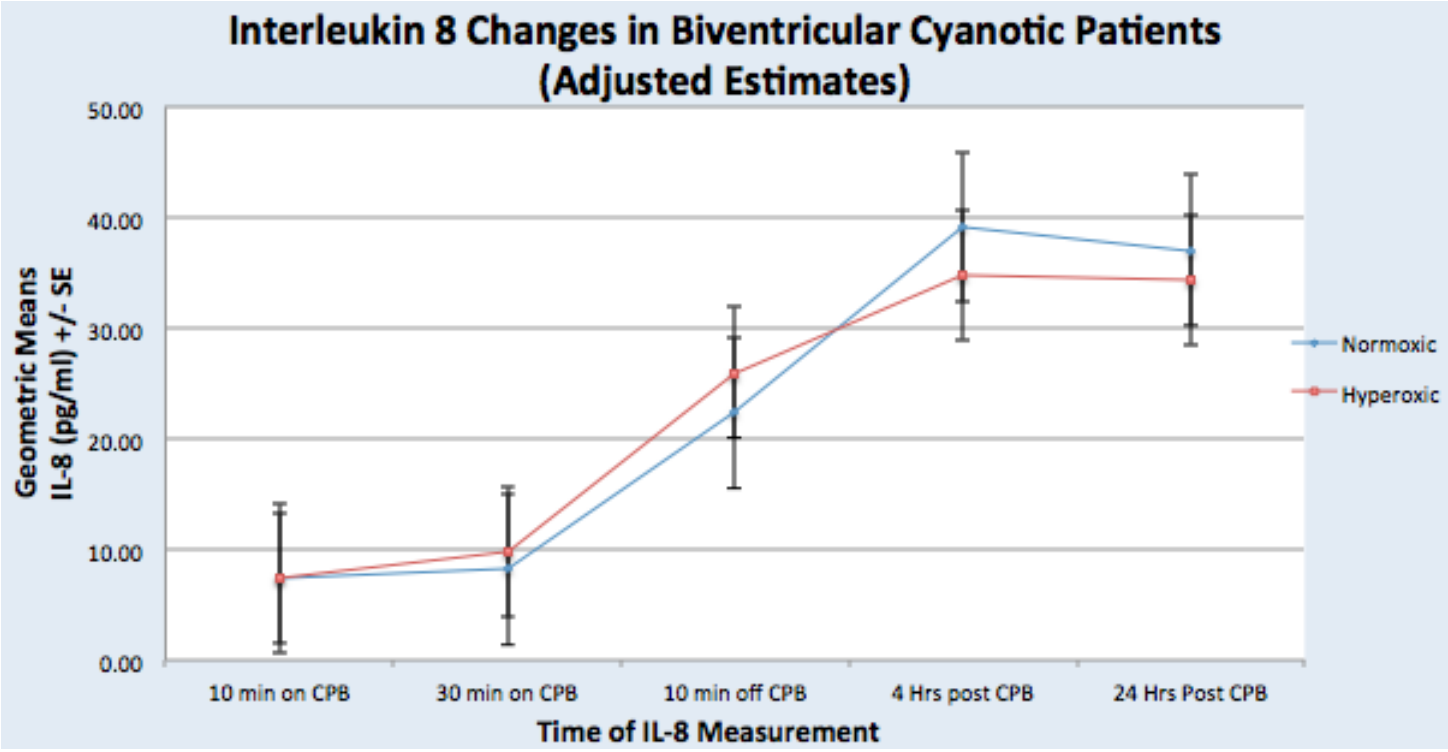

Figure 38 Interleukin-8 measurements in hyperoxic vs. normoxic groups with biventricular cyanotic pathology

4.1.3.19 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Other Inflammatory Markers (IL-10, C3a, and Cortisol) in All Groups:

In both groups, IL-10 levels rose after the initiation of CPB and remained higher than at baseline (Table 8 and Figure 39). The C3a release was greatest 10 minutes after coming off CPB and then it declined (Figure 40). In contrast, cortisol levels fell dramatically during surgery and remained lower than at baseline by 4 hours after coming off CPB (Table 8 and Figure 41).

For the above three inflammatory markers, there were no statistically significant differences between the hyperoxic and normoxic groups (P ≥ .13,Table 8).

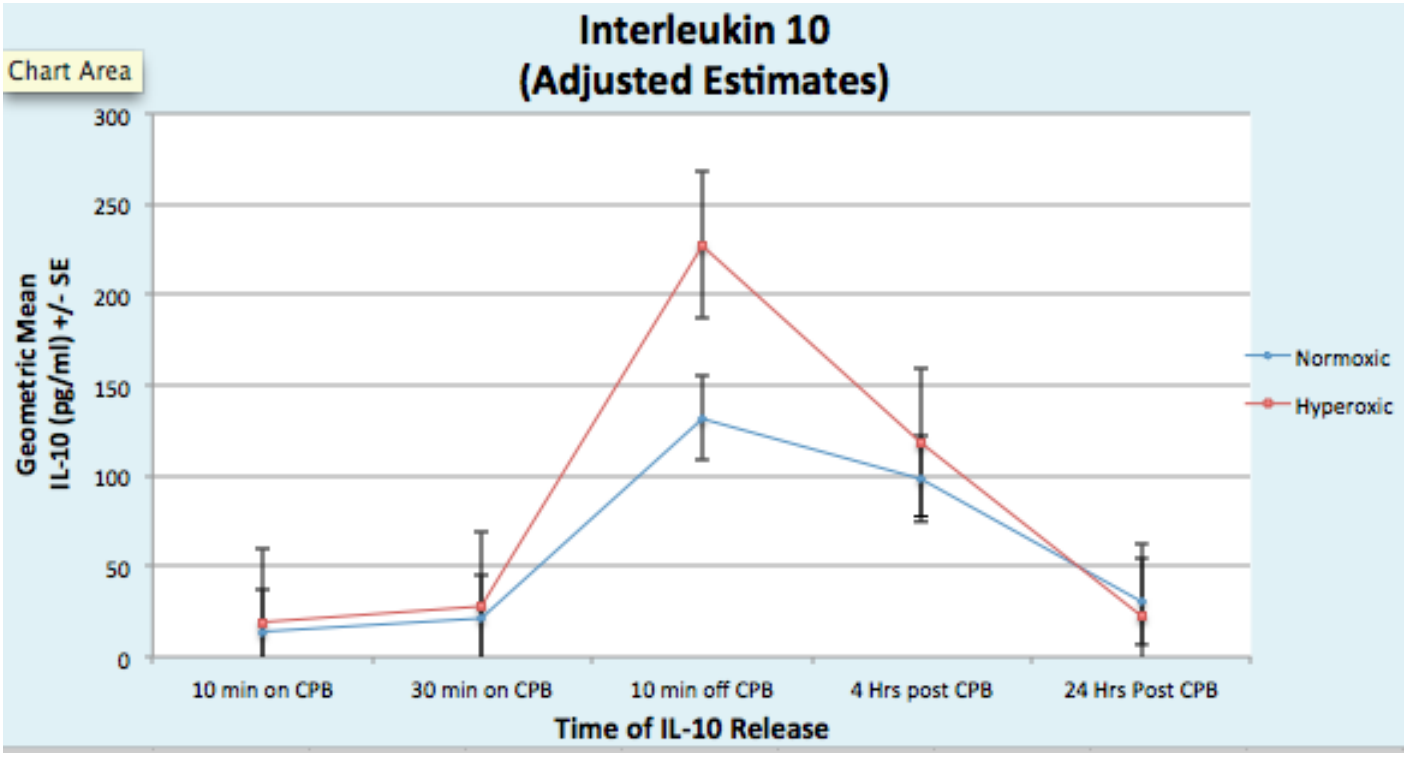

Figure 39 IL-10 release comparisons in normoxic and hyperoxic groups in all patients

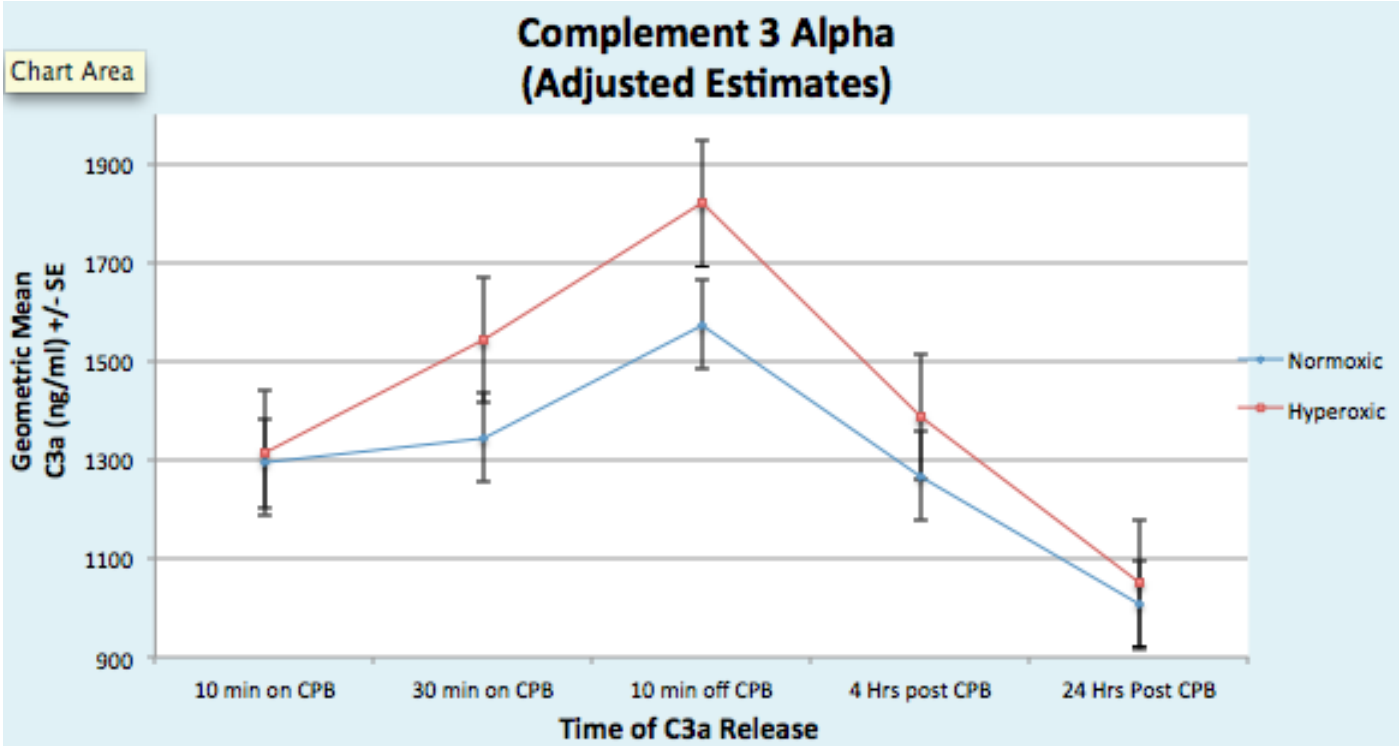

Figure 40 C3 alpha comparisons in normoxic and hyperoxic groups in all patients

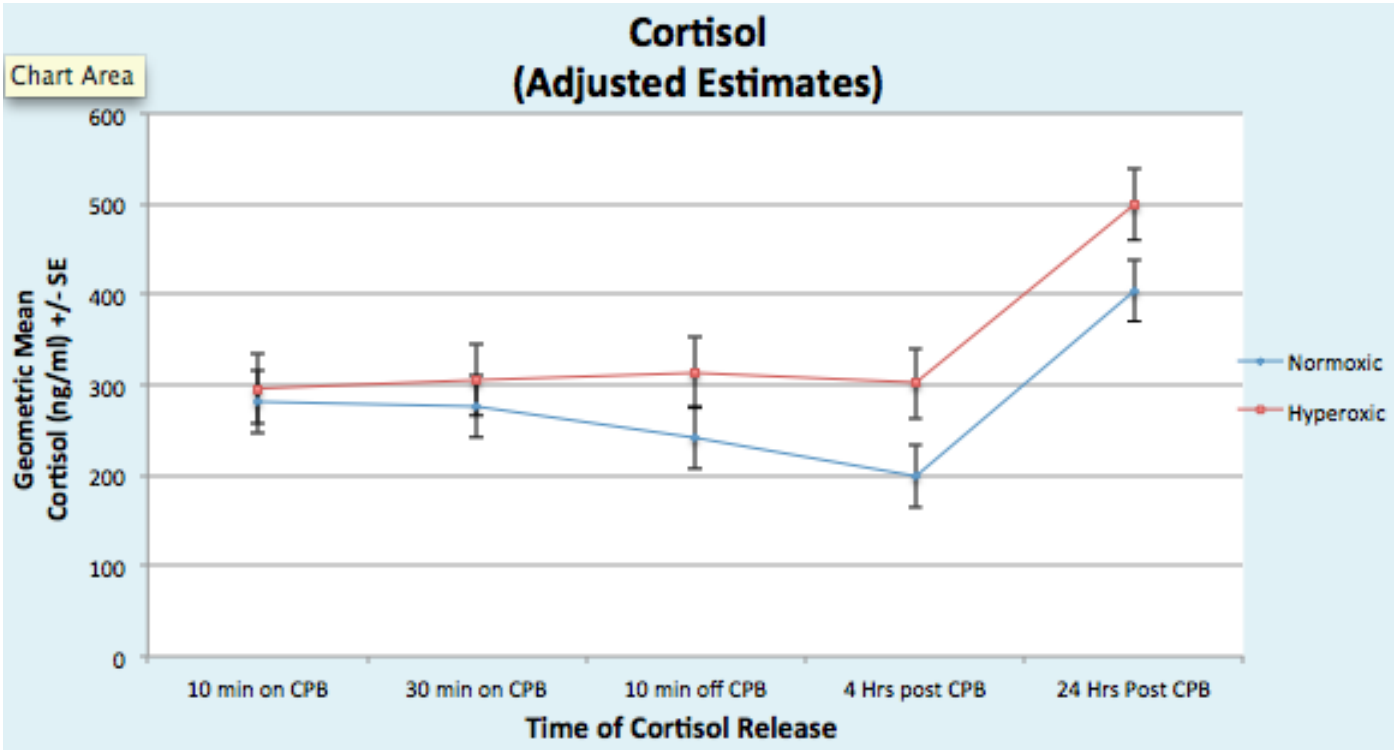

Figure 41 Cortisol level comparisons in normoxic and hyperoxic groups in all patients

4.1.3.20 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass in Release of Complement 3 alpha (C3a) in Patients With Functional Single-Ventricular Pathology:

The levels in both groups showed an initial decline by 10 minutes after the initiation of CPB (Table 11). However, the normoxic group showed a further reduction in levels by 30 minutes following initiation of CPB. It peaked 10 minutes post cessation of CPB then declined to below the pre-operative levels. In the hyperoxic group, however, the levels of C3a peaked 30 minutes post initiation of CPB and declined from then onwards (Figure 42 and Table 11). In contrast to the C3a levels for all patients, the overall levels of plasma C3a were significantly higher in the hyperoxic group compared to the normoxic group in patients with single-ventricular pathology (*ratio [normoxic/hyperoxic] = 0.87, 95% CI 0.81–0.93, P < .01*) (Table 11)

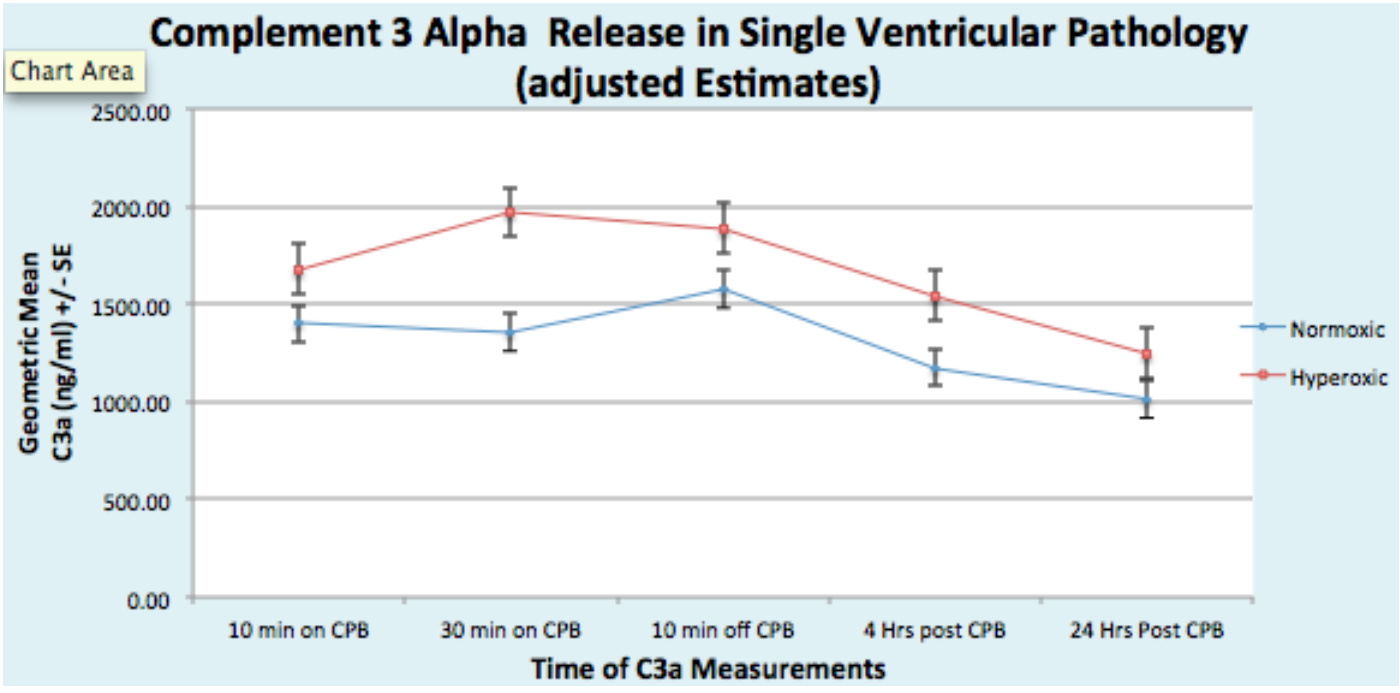

Figure 42 C3a levels in normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.21 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Complement 3 alpha (C3a) in Double-Ventricular Cyanotic Patients:

The C3a levels in both normoxic and hyperoxic groups showed a decline by 10 minutes after the initiation of CPB compared to the baseline (Table 14). The levels peaked by 10 minutes after stopping the CPB (Figure 43 and Table 14). There was no difference in release of C3a between the normoxic and hyperoxic groups in patients with biventricular cyanotic pathology *(P=0.38)*(Table 14).

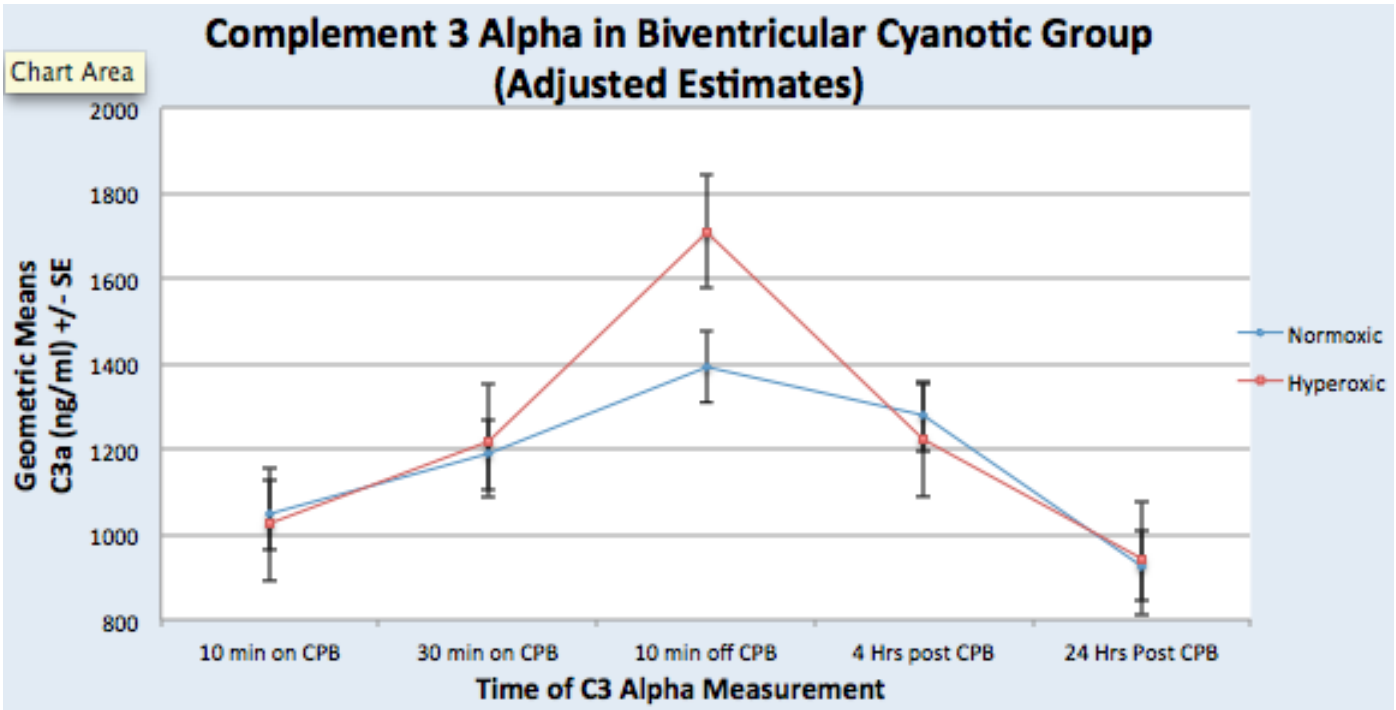

Figure 43 Measurements of C3 Alpha in normoxic vs. hyperoxic groups in cyanotic patients with double ventricular pathology

4.1.3.22 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Cortisol in Patients With Functional Single-Ventricular Pathology:

Cortisol levels in both groups showed a marked decrease compared to the baseline by 10 minutes post initiation of CPB. However at 24 hours post cessation of CPB the levels were higher than baseline (Table 11). In patients with single-ventricular pathology, the overall plasma cortisol levels were significantly higher in the hyperoxic group in contrast to the normoxic (*ratio [normoxic/hyperoxic] = 0.91, 95% CI 0.83–0.99, P = .04*) (Table 11 and Figure 44).

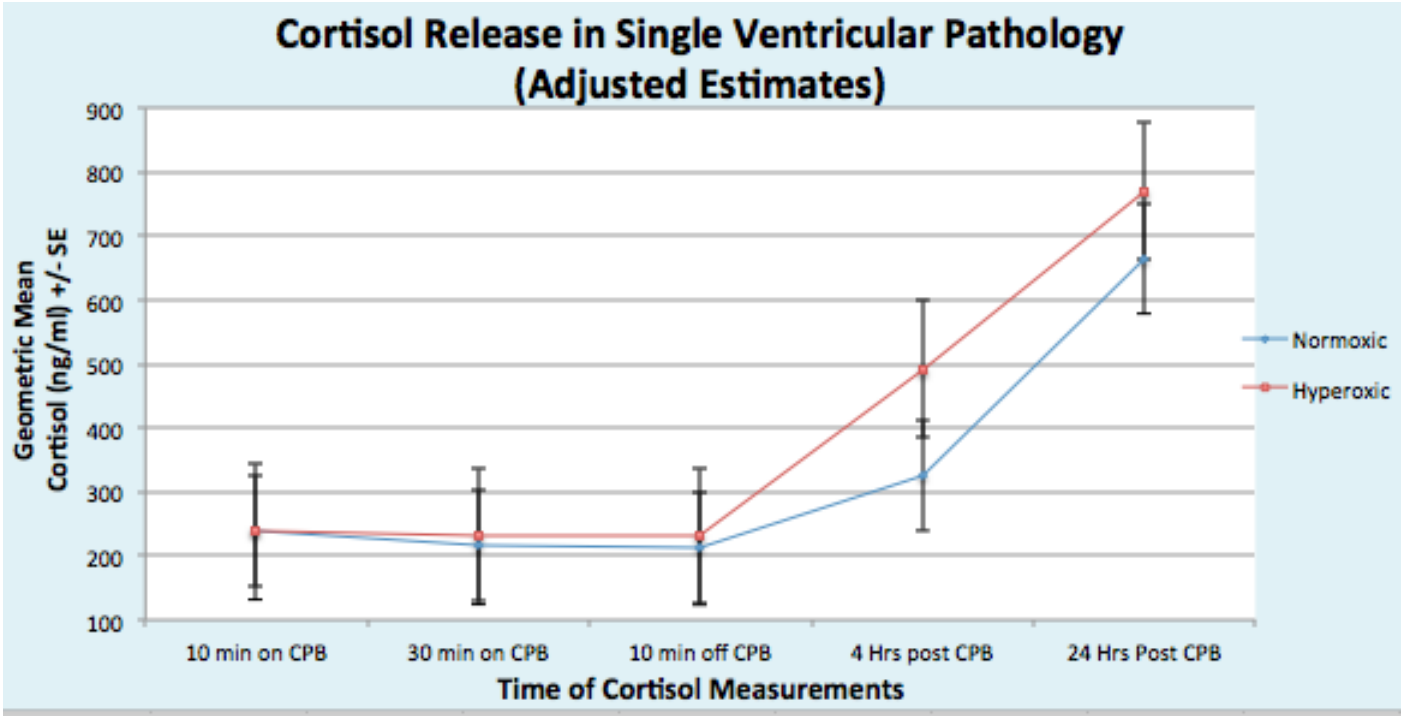

Figure 44 Comparison of cortisol release between normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.23 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Cortisol in Double-Ventricular Cyanotic Patients:

The serum cortisol levels in both the normoxic and hyperoxic groups showed a marked drop compared to the baseline after 10 minutes following initiation of CPB and continued to remain low until 24 hours post cessation of CPB (Table 14). The levels were at their lowest at 4 hours after stopping the CPB and the pattern of change was similar in both groups (Figure 45 and Table 14). Patients with biventricular cyanotic pathology that were randomised to the normoxic arm of this study had significantly lower cortisol levels compared to the hyperoxic arm *(ratio [normoxic/hyperoxic] = 0.85, 95% CI 0.78–0.93, P < .01)*(Table 14).

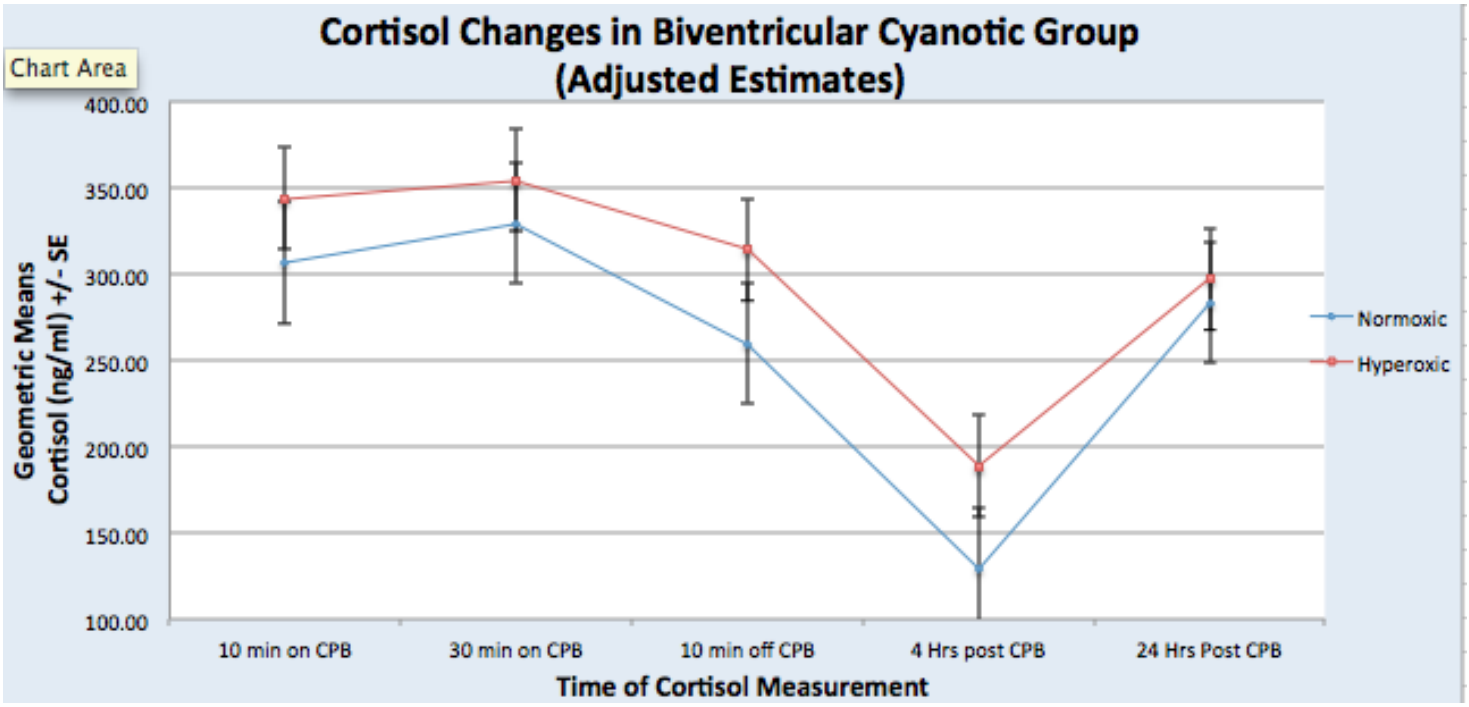

Figure 45 Normoxic vs. hyperoxic plasma cortisol level changes in cyanotic patients with double ventricular pathology

4.1.3.24 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin 10 (IL-10) in Patients With Functional Single-Ventricular Pathology:

In both groups, IL-10 levels rose significantly after the initiation of CPB compared to the baseline (Table 11). The pattern of change was similar in both normoxic and hyperoxic groups (Figure 46 and Table 11).

There was no evidence to suggest any significant difference between the normoxic and hyperoxic groups (*P = 0.1*)(Table 11).

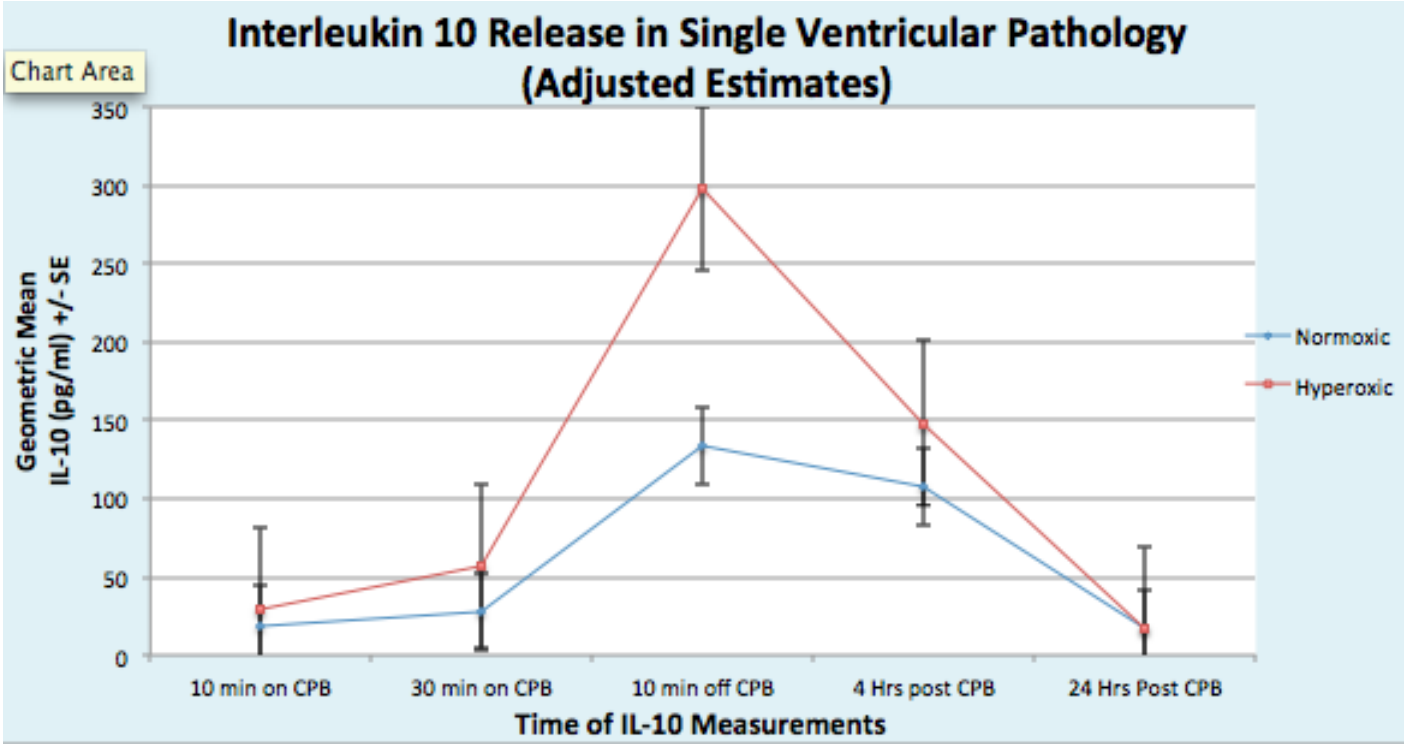

Figure 46 IL-10 level comparisons between normoxic vs. hyperoxic groups in patients with functional single ventricular pathology

4.1.3.25 Effect of Normoxic Versus Hyperoxic Cardiopulmonary Bypass on the Release of Interleukin-10 (IL-10) in Double-Ventricular Cyanotic Patients:

In both normoxic and hyperoxic groups, IL-10 levels rose significantly after the initiation of CPB compared to the baseline (Table 14). The levels peaked by 10 minutes after coming off CPB and the pattern of change was similar in both groups (Figure 47 and Table 14). Unlike the patients with single ventricular pathology, there was a statistically significant difference in the serum IL-10 changes between the normoxic versus hyperoxic patients with biventricular cyanotic pathology *(ratio [normoxic/hyperoxic] = 0.79, 95% CI 0.66–0.95, P = 0.01)*(Table 14).

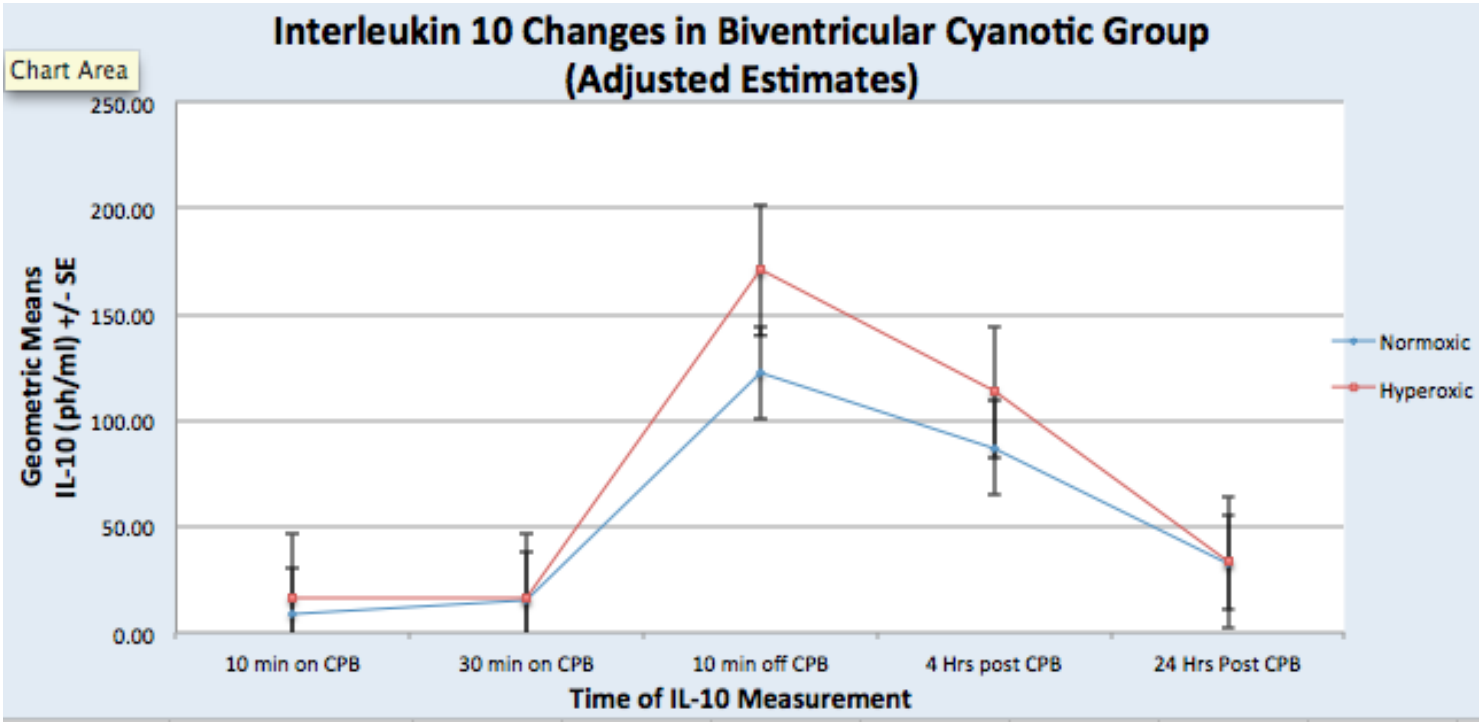

Figure 47 Interleukin-10 comparison between normoxic vs. hyperoxic groups in patients with biventricular cyanotic pathology

5 - Discussion and Conclusion

5.1 Discussion

In this study, we demonstrated that "controlled reoxygenation" on starting CPB in cyanotic patients undergoing corrective cardiac surgery is beneficial and significantly reduces the reoxygenation injury. Normoxic CPB was indeed associated with reduced oxidative stress, cerebral, myocardial and hepatic injury as well as systemic inflammatory response compared with hyperoxic CPB.

5.1.1 Myocardial Damage and Reoxygenation Injury:

5.1.1.1 Assessment of myocardial injury with Troponin-I (Tn-I)

One of the key contributors to post-operative morbidity and mortality in paediatric cardiac surgery is post-operative myocardial dysfunction. Compared to adults who undergo repair of acquired normoxic defects, the myocardial dysfunction has been found to be more severe in children [233, 378, 379]. Furthermore the response to inotropic agents in neonates is reduced when compared to adults [378, 379]. Therefore, myocardial function preservation in neonates during cardiac surgery is of great importance. A proposed strategy to prevent reoxygenation injury is to employ controlled reoxygenation during CBP.

In this study, troponin-I and 8-Isoprostane were used as a measure to assess myocardial damage and reoxygenation injury.

We showed that Troponin-I was significantly lower in the normoxic group versus the hyperoxic ($p<0.01$ Table 8). The breakdown of all patients into single-ventricular and double-ventricular showed that in single-ventricular patients, the myocardial injury was significantly higher in those who were randomised to hyperoxic group compared to the normoxic group ($p<0.01$ Table 11). However no difference was found between the normoxic versus hyperoxic groups in the biventricular patients ($p=0.31$ Table 14).

The comparison of mean troponin-I levels between biventricular and single ventricular patients showed higher troponin-I levels in the biventricular patients. (Table 11 and Table 14). This could be explained by the fact that most of the patients with biventricular pathology had ventriculotomy during surgery in order to optimise the

ventricular outflow tract. This myocardial resection may lead to a further release of troponin, resulting in a higher overall troponin levels in biventricular patients compared to the single ventricular patients. Imura *et al* have also stated this when they reported a further release of Troponin after ventriculotomy [284]. Moreover, the mean CBP time was also longer in patients with biventricular cyanotic pathology (104.32 min vs 75.26 min) (Table 7) and all of the biventricular patients underwent cardioplegic arrest (compare to only 25% of the single ventricular patients who had cardioplegic arrest). In addition the mean X-clamp time was lengthier in the biventricular group (59.45 min) compared to the 25% of the single-ventricular patients who had their aorta X-clamped (34.1 min) (Table 7). There is evidence that X-clamping can lead to a further cardiac insult which has a direct correlation to the X-clamp duration [380]. All these additional releases of troponin-I may hinder accurate assessment of myocardial injury solely related to hyperoxic injury. A larger sample size in biventricular group may be needed in order to demonstrate a more accurate finding regarding release of Troponin-I as a result of hyperoxic cardiopulmonary bypass.

5.1.1.2 Oxidative stress assessment with 8-Isoprostane

8-Isoprostane plasma levels are now considered a reliable method to assess oxidative stress. They are therefore a valuable biomarker to study the effects of oxidative injury in vivo [303]. Assays of 8-Isoprostane have revealed a role of free radicals and oxidant injury in a wide variety of pathological conditions, including ischaemia-reperfusion insult [303, 381-384]. It has been shown to be a suitable marker to measure the extent the oxidant stress has affected the myocardium during myocardial infarction [385, 386] as well as being a quantitative marker for oxidant stress during coronary reperfusion [387].

In this randomised trial, we showed that 8-Isoprostane was significantly higher in the hyperoxic group compared to the normoxic group ($p<0.01$, Table 8). This applied to both single ventricular and biventricular patients ($p=0.002$, Table 11 and $p<0.01$, Table 14). The increased 8-Isoprostane levels in the hyperoxic group suggest the direct effect of CPB oxygen levels on reperfusion injury. This finding corresponds with earlier reports indicating oxidative injury associated with hyperoxic CPB [388]. It is now evident that oxidative stress is a major part of the cellular mechanism that can result in

myocardial damage [385-387, 389]. Allen and associates demonstrated that cyanotic infants undergoing corrective cardiac surgery had a better antioxidant reserve capacity with lower levels of oxygen[14]. Similar findings were also reported by Bulutcu and co-workers [280].

The most common preoperative stress in paediatric cardiac patients is hypoxia (cyanosis) [378, 379]. Cyanotic heart is more vulnerable and less tolerant to subsequent surgical ischaemia than the normoxic heart [2, 5, 233, 378, 379, 390]. Moreover, cyanosis leads to myocardium having fewer endogenous antioxidant reserves and now there is gowning evidence (experimental and clinical) that this can make the immature cyanotic heart more venerable to oxygen induced injury when oxygen is restored [1, 4, 8]. Therefore, there are major concerns that reoxygenation injury could occur with the introduction of high levels of oxygen that can ultimately lead to cell membrane degradation through lipid peroxidation [1, 9].

Corno and colleagues [391], as well as some others, have documented experimentally that a reoxygenation injury does occur after a hypoxic insult [1, 4, 8, 233, 392]. Hirschi *et al*, in a retrospective analysis demonstrated that out of 102 neonates with respiratory failure supported with extracorporeal membrane oxygenation (ECMO), 8 patients developed severe myocardial dysfunction that was noted shortly after onset of bypass. The neonates in the cardiac dysfunction group were more hypoxic than the others [393]. Bandali and co-workers in their study, randomly allocated Yorkshire piglets to normoxia or hyperoxia under general anaesthesia. They showed that hyperoxic piglets suffered significant reductions in myocardial contractility and blood pressure. They concluded that in new-borns hyperoxia triggers oxygen free radical-mediated membrane injury together with an inability of the new-born's heart to up-regulate its antioxidant enzyme defences, leading to an impaired myocardial function and haemodynamics [394].

Work in the new-born pig heart by Ihnken and colleagues [10, 395] addressed the effects of hyperoxia on myocardium after a period of acute hypoxia or ischaemia and identified the development of significant myocardial dysfunction. It is worth noting that clinical evidence in children with acyanotic congenital heart disease also shows that hyperoxia decreases cardiac and stroke indices [396]

5.1.2 Lower Protein S100 in Normoxic CPB Suggests Less Cerebral Injury Compared to Hyperoxic CPB:

In our randomised trial, we assessed cerebral injury by measuring the serum protein S100. Protein S100 is considered one of the most specific and sensible markers of cerebral injury after cardiac surgery and is widely used in clinical trials [338].
We demonstrated that serum protein S100 was significantly higher in the hyperoxic group compared to the normoxic (Table 8) with values that peaked 10 minutes after cessation of CPB. S100 in both groups returned to preoperative levels 24 hours after the operation (Figure 27). This applied to both single ($p<0.01$ Table 11) and biventricular patients ($p<0.01$ Table 14). These findings suggest a less detrimental effect of normoxic CPB on the brain when compared to hyperoxic CPB. In support of our findings, there is clinical evidence of the effects of reoxygenation injury on cerebral function reported by Matheis and colleagues [397]. In their observational study, the authors demonstrated that uncontrolled hyperoxic reoxygenation on CPB for surgical correction of congenital heart defects was associated with higher S100 levels in cyanotic infants compared with acyanotic patients undergoing comparable operations.

In recent years, hypoxia and reoxygenation have emerged as very important mechanisms of cerebral injury. Several studies have indicated that hyperoxia exacerbates post-ischaemic reperfusion injury in brain as well as kidney and myocardium [19, 398-401]. Among patients admitted to the ICU following resuscitation from cardiac arrest, arterial hyperoxia was independently associated with increased in-hospital mortality compared with either hypoxia or normoxia [402]. In past few years, experimental studies revealed that neonates suffering from respiratory distress who were treated with higher levels of oxygen in Neonatal Intensive Care Units, developed oxidative and nitrative stress. This led to an increased neuronal and oligodendroglial apoptosis in the developing brain [403-405]

In an animal study, Zwemer *et al* examined the effects of normoxic ($F_iO_2 = 0.21$), versus hyperoxic ($F_iO_2 = 1.0$) resuscitation on the neurological outcome following 9 minutes of normothermic cardiac arrest. They showed hyperoxically resuscitated dogs sustained significantly worse neurological deficit at 12 and 24 hours than the normoxically

resuscitated ones. They concluded that hyperoxic F_iO_2s could contribute to a further exacerbation of neurological dysfunction [401].

Kurul *et al* similarly in a separate study have indicated that exposure to hyperoxia in infant rats leads to extensive apoptotic degeneration in the cortex and white-matter of the developing brain [406]. In a different study, Yis *et al* studied the effects of high oxygen on brain tissue. They examined Wistar rat pups, which were exposed from birth until day 5 to 21% (normoxic) or 80% (hyperoxic) oxygen. They reported that neuronal densities of the different areas of the brain were significantly decreased in the hyperoxic group and that hyperoxia induced cell death in the developing rat brain. They have claimed that this may be one of the important mechanisms that cause motor and cognitive impairment in later life of premature infants [407].

Diringer and co-workers used positron emission tomography (PET) to study the impact of hyperoxia on patients with acute severe traumatic brain injury. They found that hyperoxia does not increase oxygen utilisation and has no improvement on cerebral oxygen metabolic rate [408]. Guo *et al* reported that astrocyte viability was greatly decreased by hyperoxia compared with normoxic controls in an in vitro model of hypoxia-reoxygenation [409]. Sher and Hu [327] also showed in an in vitro cell model that gradual reoxygenation after prolonged hypoxia improves neuronal survival compared with rapid reoxygenation and delays the manifestations of metabolic dysfunction. Their findings are also consistent with the concept that a period of relative hyperoxia may contribute to hypoxia-induced neuronal injury. Similarly, Stauton and co-workers have shown that hypoxia–reoxygenation causes endothelial dysfunction in intraparenchymal cerebral arterioles by impairing endothelium-dependent dilation of micro-vessels, which in turn may decrease oxygen delivery and increase neuronal injury [328].

5.1.3 Serum Alpha GST Assay Suggests Less Hepatic Injury in Normoxic CPB when Compared to Hyperoxic CPB:

In this study liver injury was assessed by measurements of the plasma Alpha Glutathione S-Transferase (άGTs).

The άGTs are a group of cytosolic proteins that constitute up to 2-5% of the soluble protein in hepatocytes [316]. The baseline level of άGTs in serum is extremely low, and as such it is easy to monitor any increases that may occur. άGT is a very sensitive and specific biomarker of hepatocyte injury [326]. It is unaffected by muscle injury and other factors that can cause elevated transaminase levels [316]. An elevated άGT level indicates hepatocyte injury even when most other markers are normal. A normal serum άGT level can almost exclude acute hepatocyte injury [316].

Our results revealed that in both single ventricular and biventricular patients, the άGT levels were significantly higher in the hyperoxic group compared to the normoxic group ($p<0.01$ Table 11 and Table 14). The levels peaked by 4 hours post termination of CPB in both groups (Figure 31 and Figure 32)

Hepatic reoxygenation injury has been demonstrated in experimental animal models of rats undergoing induced hypoxia for 60 minutes followed by 25 minutes of reflow[314]. It was reported that during reoxygenation of perfused rat liver, there was an increased oxyradical production leading to liver injury [315]. Caraceni *et al* reported that there is a correlation between liver reoxygenation injury and oxygen free radical (OFR) generation. They demonstrated this by submitting rat hepatocytes to 2.5 hours of anoxia followed by 2 hours of reoxygenation. From this, they isolated liver parenchymal cells that generated measurable amounts of superoxide anions [410]. In a different study, Bhogal and co-authors demonstrated that endogenous reactive oxygen species (ROS) production by mitochondria and nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase) could trigger human hepatocyte apoptosis and necrosis during hypoxia and hypoxia-reoxygenation, which may therefore play an important role in hepatic injury [411]. Jaeschke *et al* concluded that ischaemia-reperfusion injury, even in normothermic level, is an important determinant in the pathogenesis of liver damage

occurring during surgical procedures [412]. Rogers *et al* have reported; an early increases in 5-lipoxygenase (5-LOX) and cyclooxygenases-2 (COX-2) protein levels as well as increases in total hydroxyeicosatetraenoic acid (5-HETE) and prostaglandin levels in the liver tissues of hyperoxia-exposed pups. They indicated that exposure to higher levels of oxygen in newborn mice alter the hepatic eicosanoid metabolism which could play a role in hyperoxic liver injury [413].

There is little in the literature about liver injury in cyanotic patients undergoing corrective cardiac surgery. Ascione *et al* from Bristol Heart Institute, in a randomised trial, demonstrated higher serum levels of ALT and AST post coronary artery bypass graft (CABG) using cardiopulmonary bypass machine versus off pump CABG. One can conclude that the CPB machine on its own can have detrimental effect on the liver. However in our study, there was clearly a significant difference in serum Alpha GST levels between the normoxic and hyperoxic groups suggesting that hyperoxic CPB can have a more severe detrimental effect on the liver compared to normoxic.

To the best of our knowledge, this represents the first clinical evidence of a reduced hepatic injury when a strategy of controlled reoxygenation CPB is adopted in cyanotic children undergoing cardiac surgery.

5.1.4 Systemic Inflammatory and Stress Response Assessment in Normoxic vs Hyperoxic CPB:

A systemic inflammatory response can occur following various events including reperfusion injuries [345]. A devastating consequence of reperfusion is the development of remote organ injury and Multi Organ Dysfunction Syndrome (MODS) [414]. Some of the risk factors for MODS is CPB and aortic cross-clamping [415]. This can lead to pulmonary, hepatic, renal, gastrointestinal, myocardial and CNS dysfunction [416]. Nevertheless it might be possible to minimise these injuries with a controlled reoxygenation strategy.

In this randomised trial we studied the systemic inflammatory response by measuring the plasma levels of interleukin-6 (IL-6), interleukin-8 (IL-8), Complement C3 alpha (C3a), Interleukin-10 (IL-10) and cortisol.

5.1.4.1 Release of Lower Levels of Interleukin 6 (IL-6), Interleukin 8 (IL-8) and Complement C3 alpha (C3a) in Normoxic Cardiopulmonary Bypass Group:

In this trial we found that there was a rise in IL-6, IL-8 and C3a post initiation of cardiopulmonary bypass. This confirmed the previous reports on IL-6 and IL-8 liberation during CPB [417-420]. We first looked at all patients and found that the normoxic group had an overall lower IL-6 and IL-8 levels compared to the hyperoxic group ($p < 0.01$ Table 8). Our results confirmed the work of Bulutcu *et al*, who demonstrated cyanotic patients undergoing corrective surgery with FiO_2 of 0.21 (21%) had a lower level of IL-6 compared to CPB with FiO_2 of 1.0 (100%) [280]. However we did not detect any difference in the release of C3a between normoxic versus hyperoxic groups for all patients ($p=0.25$ Table 8).

We then looked at patients with single ventricular and double ventricular pathology separately. We found that patients with single ventricular pathology in the normoxic group also had a lower IL-6 ($p<0.01$) and IL-8 ($p<0.01$) levels compared to the hyperoxic group (Table 11). Interestingly, we also found that the release of C3a was lower ($p<0.01$ Table 11) in patients with single ventricular pathology who were randomised to the normoxic arm of this study. However no difference in IL-6, IL-8 and C3a levels was identified in patients with biventricular pathology ($p=0.3$, $p=0.61$ and $p=0.38$ Table 14).

This discrepancy in IL-6, IL-8 and C3a findings between single ventricular and double ventricular patients could be explained by the fact that the mean CPB time in double ventricular patients was longer compared to the mean CPB time in single-ventricular patients (Table 7). Moreover only 25% of the patients in the single-ventricular group had their aorta cross-clamped (mean cross calmp time = 34.1 min.) and the other 75% had their surgery with out cross clamp. In contrast, all patient in the biventricular group had their surgery with the aorta cross-clamped (mean X-clamp time = 59.45 min.) (Table 7). It is arguable that longer CPB and cross clamp time in the biventricular group can further trigger systemic inflammatory response and lead to an additional release of

IL-6, IL-8 and C3a. This may make the assessment of increased levels of IL-6, IL-8 and C3a purely related to normoxic vs. hyperoxic CPB less accurate. A larger sample size may be able to support this theory.

When we earlier reported our preliminary results for a smaller sample size (the first 67 patients of this study) [3], although we showed higher levels of IL-6 and IL-8 for the hyperoxic group, we did not find any statistically significant difference. In this research however, not only have we confirmed our earlier findings by showing higher levels of IL-6 and IL-8 in the hyperoxic group, but we have also shown that these findings are statistically significant (Table 8). This could be the result of 12 additional patients to earlier sample size (79 compared to 67), which gives a higher power for further statistical analysis.

Interleukin 6 (IL-6) is produced by several kinds of cells, such as macrophages, fibroblasts, lymphocytes T & B as well as endothelial cells, vascular smooth muscle cells and renal mesangial cells [421-425]. It is primarily involved in the regulation of immune and inflammatory responses [426]. It functions not only as a B and T-cell–stimulating agent [423, 427], but also as a hepatocyte-stimulating factor inducing the production of a series of acute-phase proteins [428]. Elevated levels of serum IL-6 have been demonstrated in inflammations resulting from injuries induced by reperfusion post hypoxia/ischaemia [426, 429-431]. Some studies suggest that the amount of increase in the IL-6 levels observed after insult may be related to the extent of injury[432]. There is now good evidence that hypoxia-reperfusion leads to a significant increase in the expression of IL-6 in many organs including the brain [433], hind limb [434], gut [435], kidneys [426] and myocardium [436]. Progression of myocardial dysfunction following cardiac hypoxia-reperfusion insult could be attributed to the IL-6 derived from the injured cardiac myocytes [437]. Yamauchi-Takihara *et al* showed that the incubation of rat cardiac myocytes under hypoxic conditions for 4 hours induced a significant increase in the production of IL-6 compared with normoxic conditions. Furthermore, 2 hours of hypoxic stress followed by reoxygenation for 2 hours, significantly augmented the production of IL-6 by cardiac myocytes [437]. In a different study, Finkel *et al* reported the negative inotropic effect of several recombinant cytokines, including IL-6, on the

hamster papillary muscle. They stated that the direct negative inotropic effect of cytokines is mediated through a myocardial nitric oxide synthase [438].
Hypoxic stress and its association with augmented IL-6 production in cardiac myocytes would support the role of IL-6 not only as a possible mediator in neutrophil migration and activation but also in progression of myocardial dysfunction following hypoxia-reperfusion injury [437].

Interleukin-8 (IL-8) is also another proinflamatory mediator, which belongs to a family of chemotactic cytokines and has been described as a neutrophil/ T cell activating protein [439-442]. It is produced in response to ischaemia-reperfusion injury [443, 444] and there is a strong correlation between IL-8 expression and myocardial injury from oxidative stress [441]. IL-8 production is also detected after reperfusion of different hypoxic tissues such as lung, kidney and brain [208, 366, 445-447]. It stimulates the binding activity of CD11b/CD18 on human neutrophils and thus plays a critical role in the neutrophilic invasion into the damaged tissue [448]. Neutrophils have critical involvement in injuries induced by reperfusion. It has been suggested that the essential mechanism of neutrophilic invasion into areas damaged by ischaemia/hypoxia is by attachment of neutrophils onto endothelium through adhesion molecules and migration by chemotactic factors [449]. Boyle *et al* provided evidence that inhibition of IL-8 prevents the extension of myocardial injury after reperfusion [441].

Increased levels of complement 3 alpha may also be seen as a response to reperfusion injury [450]. Reperfusion injury can result in release of complement C3 in various organs, such as myocardium, lungs, kidney, intestine and striated muscle [416, 451-454]. There are numerous studies that have reported that the effects of reperfusion injury have been minimised by inhibition of complement C3 [450, 455-458].

5.1.4.2 Higher Levels of Interleukin 10(IL-10) in the Hyperoxic CPB Group With Biventricular Pathology and the Implications of This on Inflammatory Response:

In this randomised study we showed that IL-10 levels increase after CPB. This confirms the findings of Seghaye *et al* [459]. Although the IL-10 levels were higher in the hyperoxic arm for all patients and those with single ventricular pathology, we did not find any statistically significant differences (p=0.13 and p=0.1 respectively; Table 8 and Table 11). However in the biventricular group the overall IL-10 levels were higher in the hyperoxic arm compared to the normoxic arm, which was statistically significant (p=0.01 Table 14).

IL-10 plays a complex role in the immune system. The major activities of IL-10 are to inhibit macrophages from producing cytokine and to suppress their accessory functions during T-cell activation. IL-10 also inhibits IL-1 and TNF production. This plays a crucial role to the anti-inflammatory activities of IL-10 [345, 460, 461]. Therefore IL-10 is widely considered as an immunosuppressive and anti-inflammatory cytokine. Hess *et al* reported that administration of exogenous IL-10 decreased pulmonary complications associated with reperfusion injury after repair of thoraco-abdominal aortic aneurysm (TAAA) [349]. There are also reports that endogenous interleukin-10 has a protective effect against early lung reperfusion injury [462].

Engles *et al*, in an animal study, showed a lower TNF-α rise in rats that had ischaemia-reperfusion with exogenous IL-10 versus ischaemia-reperfusion alone. They reported that exogenous IL-10 attenuated both local and distant organ injury after hind-limb ischaemia-reperfusion [463].

Yang *et al* in separate study subjected wild-type and IL-10–deficient mice to myocardial ischaemia-reperfusion. They reported a significant production level of IL-10 in wild-type mice at 2 to 6 hours post myocardial reperfusion. The genetic deletion of IL-10 augmented the infiltration of neutrophil into the reperfused tissues at 6 hours post reperfusion and resulted in a larger size infarct and myocardial necrosis. Additionally, in the absence of IL-10, there was an enhancement of the overall inflammatory response. They stated that following 24 hours of reperfusion, in the IL-10–deficient

mice, the mortality rate was 75%, whereas no mortality was associated with the wild-type mice. They concluded that endogenous IL-10 inhibits the production of TNF-α as well as NO, and serves to protect the hypoxic-reperfused myocardium [464].
Various other studies have demonstrated a direct correlation between endogenous IL-10 levels and the severity of insult to the body (systemic inflammation) [465-472]. This is possibly a mechanism to minimise the extent of inflammatory response.

The findings of our study indicate the possibility that hyperoxia induces further insult to the body compared to normoxia and therefore the body compensates by generating higher levels of IL-10 to minimise the inflammatory damage. Further studies may have to be carried out to confirm this conclusion.
It is worth considering that a study with a larger sample size could demonstrate results with a significant statistical difference in patients with single ventricular pathology too.

5.1.4.3 Measurement of Serum Cortisol Levels in Normoxic versus Hyperoxic Cardiopulmonary Bypass Groups:

The production of cortisol, a primary glucocorticoid secreted by the adrenal cortex is increased by stress; therefore, cortisol may be used as a biomarker of stress [369-371]. Increased cortisol levels may be seen as a response to reperfusion injury [414, 415, 450, 473, 474] and it has been reported that the magnitude and duration of the hormonal (cortisol) response to stress correlate with the extent of the injury [475].

In this randomised trial we demonstrated that; patients with single ventricular and double ventricular pathology who were randomised to the hyperoxic arm of this study, had higher levels of cortisol when compared to the normoxic group. ($p=0.04$, $p<0.01$, Table 11 & Table 14).

In general, we showed a rise in biochemical markers for systemic inflammatory and stress response following initiation of CPB in cyanotic children. This corresponds with previous studies indicating similar inflammatory and stress response during paediatric cardiac surgical procedures [417, 476-478]. In addition to that, we also showed

that the overall inflammatory response was higher in the hyperoxic group. This was more noticeable in the single-ventricular patients.

5.2 Clinical Implication and Conclusion:

The above findings clearly signify the beneficial effects of normoxic versus hyperoxic CPB on the heart, brain and liver as well as inflammatory and systemic stress response in cyanotic patients undergoing corrective cardiac surgery. These findings were more prominent in patients with single ventricular pathology. One may conclude that single ventricular patients may even get a greater benefit from the advantages of normoxic CPB. This argument can be supported by the fact that IL-6, IL-8, C3 alpha, and Troponin-I were all lower in single-ventricular cyanotic patients who were randomised to normoxic group when compared to hyperoxic. This may potentially have an important implication on the surgical outcome of single-ventricular patients.

The findings of Finkel *et al* [438], Yamauchi-Takihara *et al* [437] and Hovels-Gurich *et al* [417], that showed IL-6 and IL-8 had a negative inotropic effect on myocytes as well as properties leading to myocardial dysfunction. Lower Tn-I levels as well as IL-6 and IL-8, in the normoxic group, may support the argument that normoxic CPB can have a reduced detrimental effect on the myocardium in patients with single-ventricular pathology.

We earlier reported our preliminary results for a smaller sample size (67 patients) [3]. In that paper we compared normoxic to hyperoxic CPB for cyanotic patients undergoing corrective cardiac surgery. However, we did not carryout a further sub-analysis to compare the single and double ventricular pathology separately. Interestingly in our earlier report, we did not find any statistically significant difference in the release of inflammatory markers (IL-6, IL-8, IL-10, C3a and cortisol) between normoxic and hyperoxic groups. However, in this study we have demonstrated differences in some of the inflammatory markers in the normoxic versus hyperoxic groups that are statistically significant. This discrepancy in results could be explained by a higher number of patients in this study (79 patients) versus the earlier report (67 patients), which adds

more statistical power to this research. It may be possible that with a larger sample size, further differences could be demonstrated in the normoxic versus hyperoxic CPB.

This randomised trial provides direct evidence that an unintended oxygen-mediated injury occurs in cyanotic patients with the initiation of CPB, resulting in myocardial, cerebral, and hepatic injury as well as triggering systemic inflammatory response. Using a novel and simple CPB strategy of controlled reoxygenation can reduce the risk of reoxygenation injury. This strategy is simple and can be incorporated at no additional risk into the operative management, as long as the perfusionist is familiar with the technique and the equipment is appropriate. It does not interfere with the surgical procedure and, by limiting oxidative stress and reoxygenation injury in this very high-risk group of patients, it might lead to a reduction in morbidity and mortality.

5.3 Limitations:

Although a sample size of 40 patients per group was calculated to detect an effect size of 0.5 or more with 90% power for this study, however a larger sample size could have provided results with more certainty particularly with regards the releas of cardiac troponin I and inflamatory markers in the biventricular group. One other limitation of this study was the lack of assessment of these patients for clinical outecomes such as survival, assessment of myocardial function and symptom evaluation (such as NHYA classification).

However, this trial provides a solid foundation to further explore the clinical outcomes such as survival, ventricular function, growth and cardio-repiratory symptoms (NYHA) in cyanotic patients undergoing cardiac surgery with normoxic versus hyperoxic cardiopulmonary bypass.

7 - References

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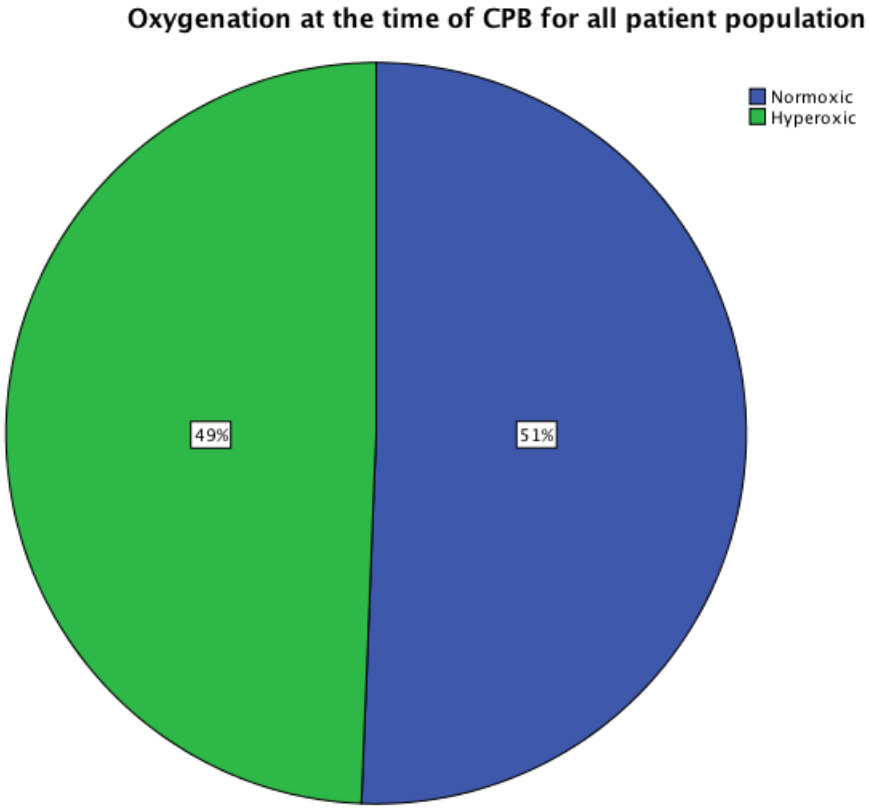
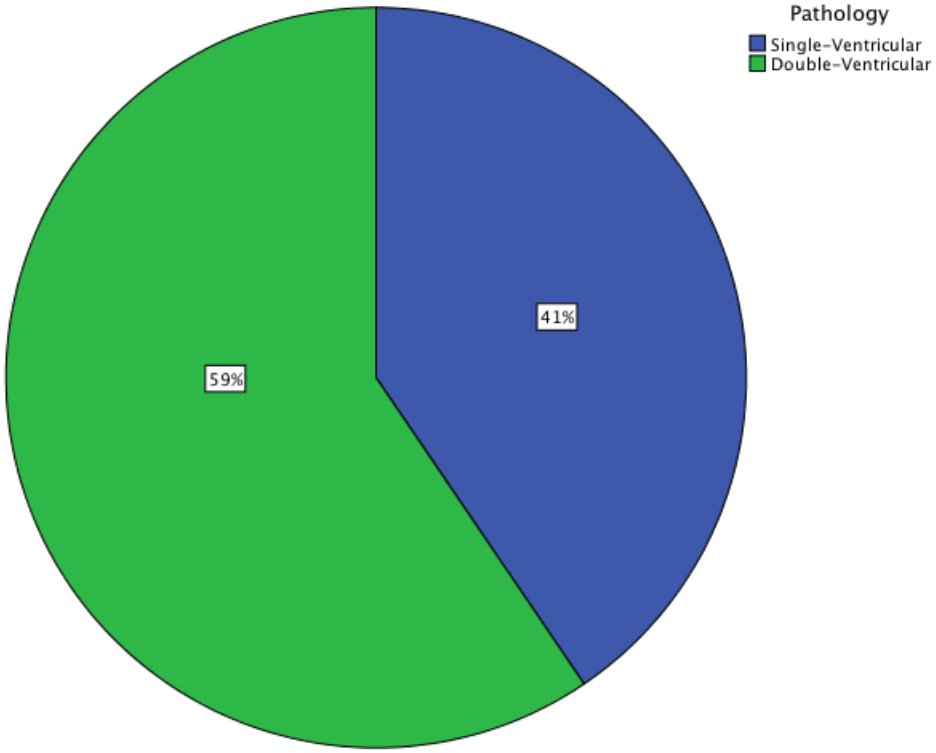
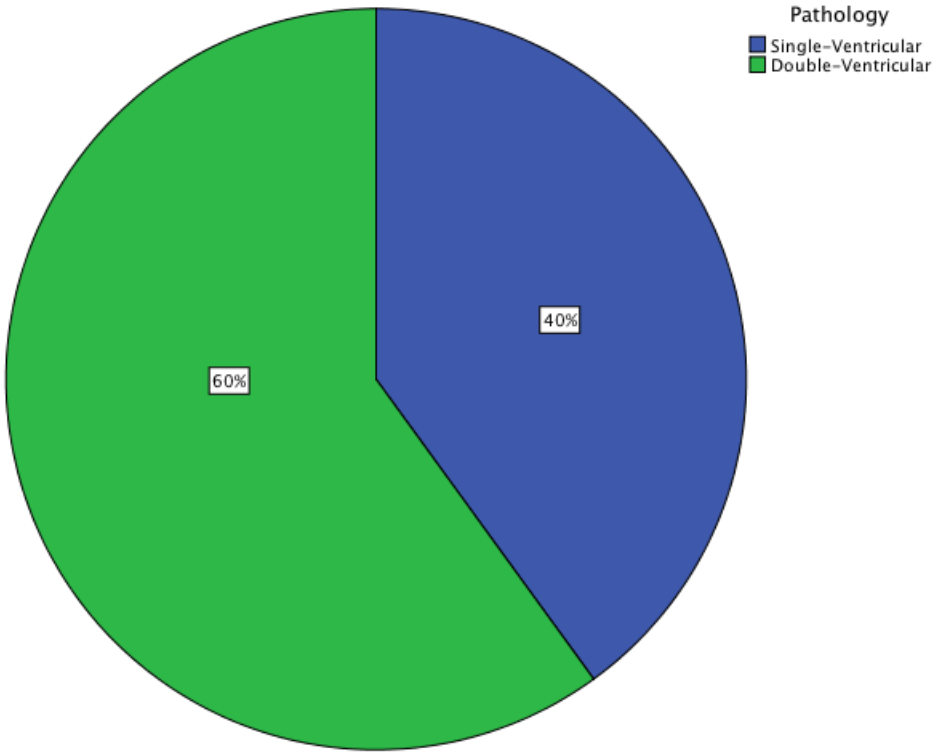
8 - Appendix


Oxygenation at the time of CPB for all patient population
Normoxic
Hyperoxic
49%
51%

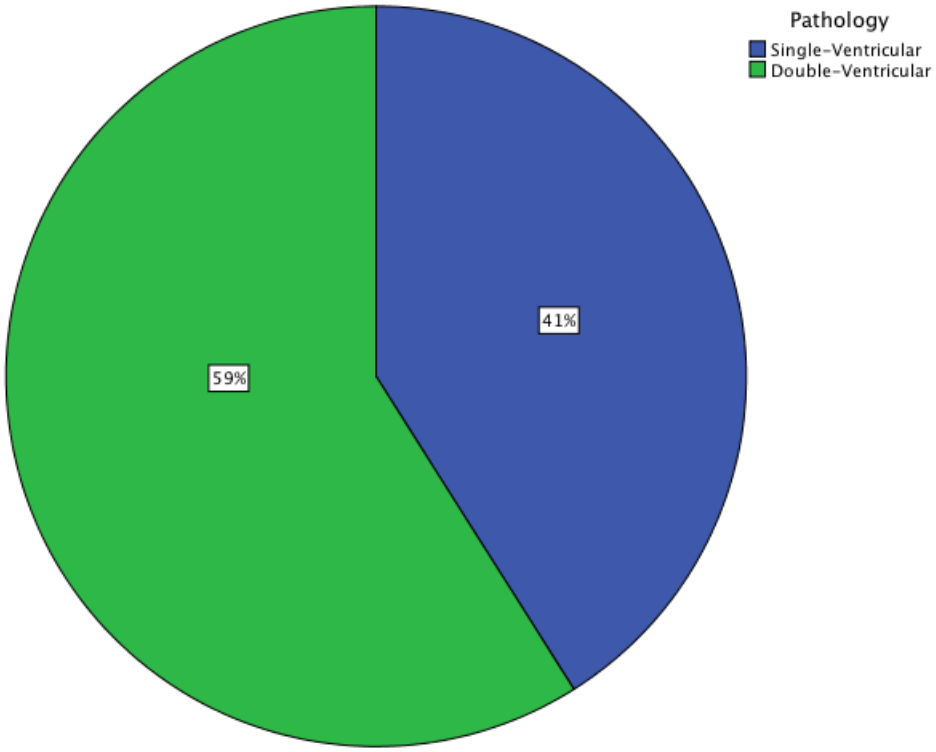
Ratio of single and double-ventricular patients



Single and double-ventricular ratio in normoxic group

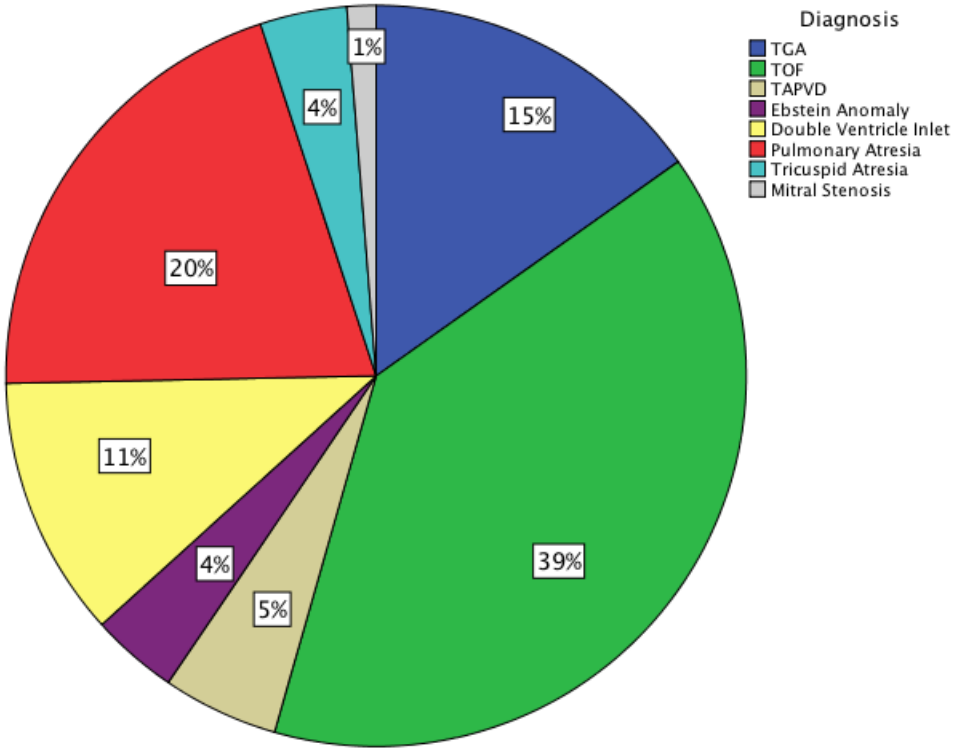


Single and double-ventricular ratio in the hyperoxic group

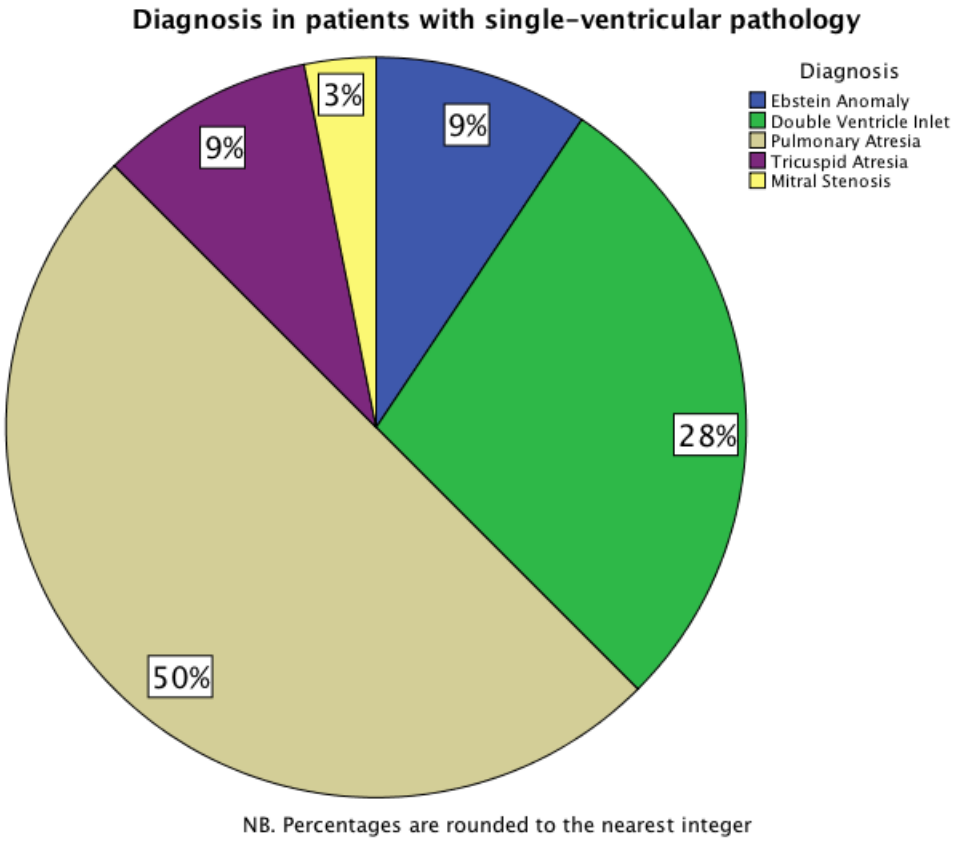


N.B Percentages are rounded to the nearest integer

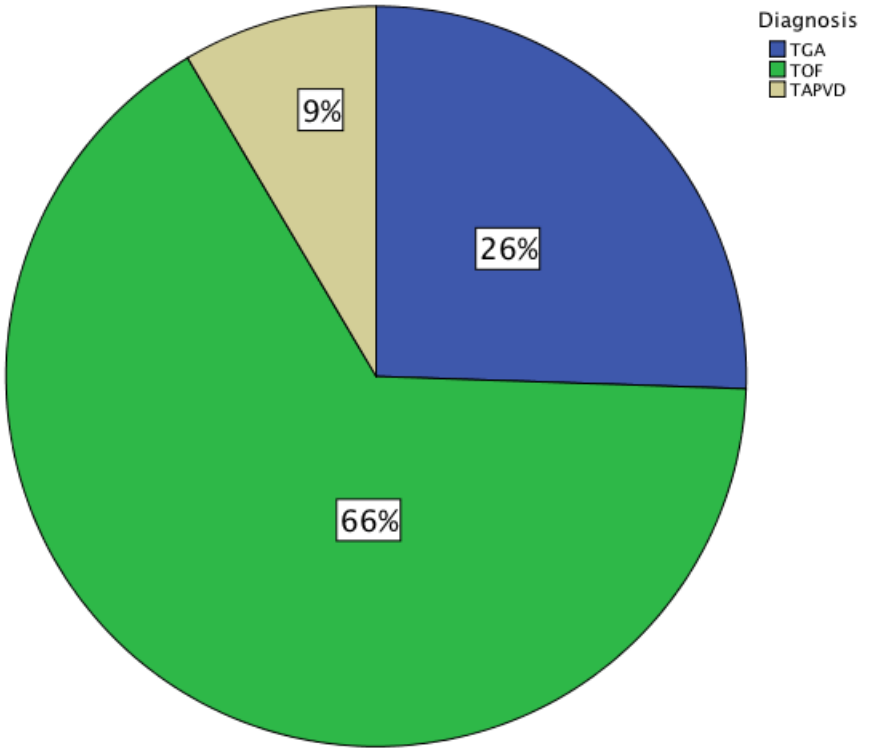
Diagnosis in all patients



NB. Percentages are rounded to the nearest integer

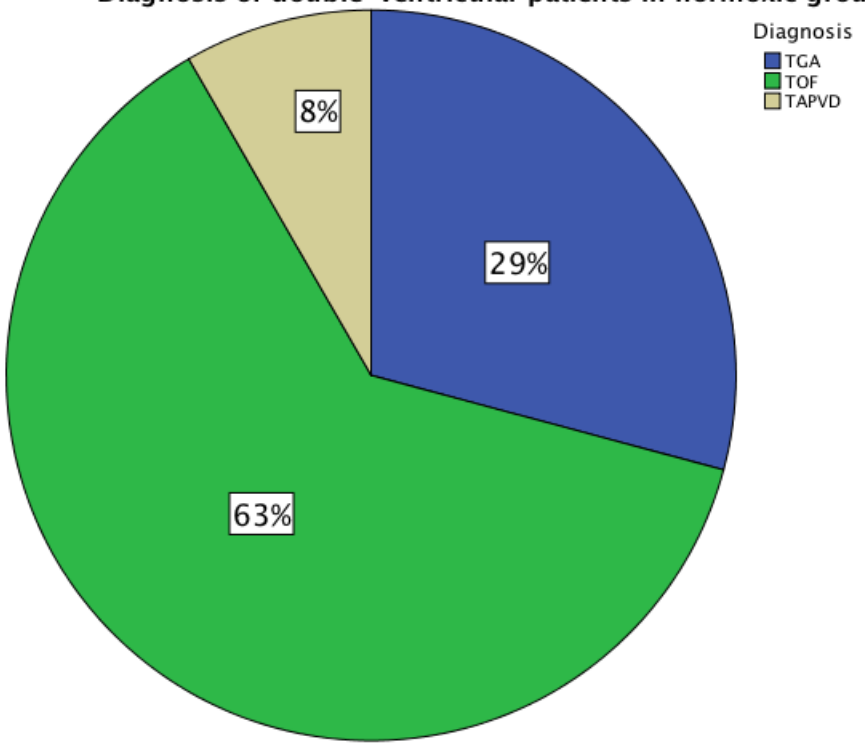

Diagnosis in patients with single-ventricular pathology
Diagnosis
Ebstein Anomaly
Double Ventricle Inlet
Pulmonary Atresia
Tricuspid Atresia
Mitral Stenosis
9%
28%
50%
9%
3%
NB. Percentages are rounded to the nearest integer

Diagnosis in patients with double-ventricular cyanotic pathology



NB. Percentages are rounded to the nearest integer

Diagnosis of double-ventricular patients in normoxic group



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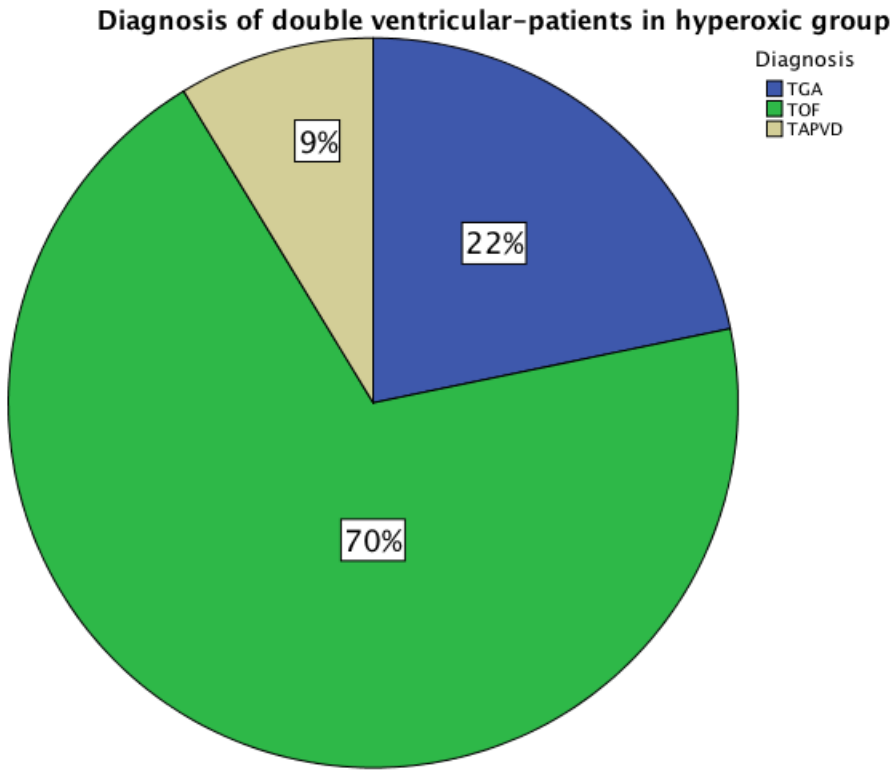

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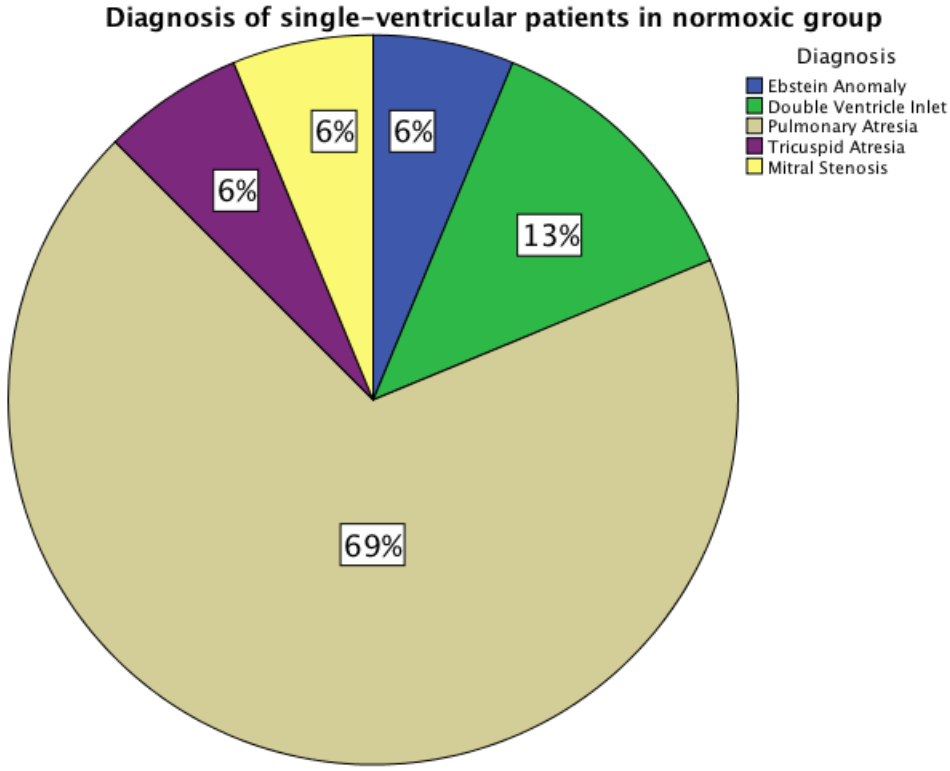

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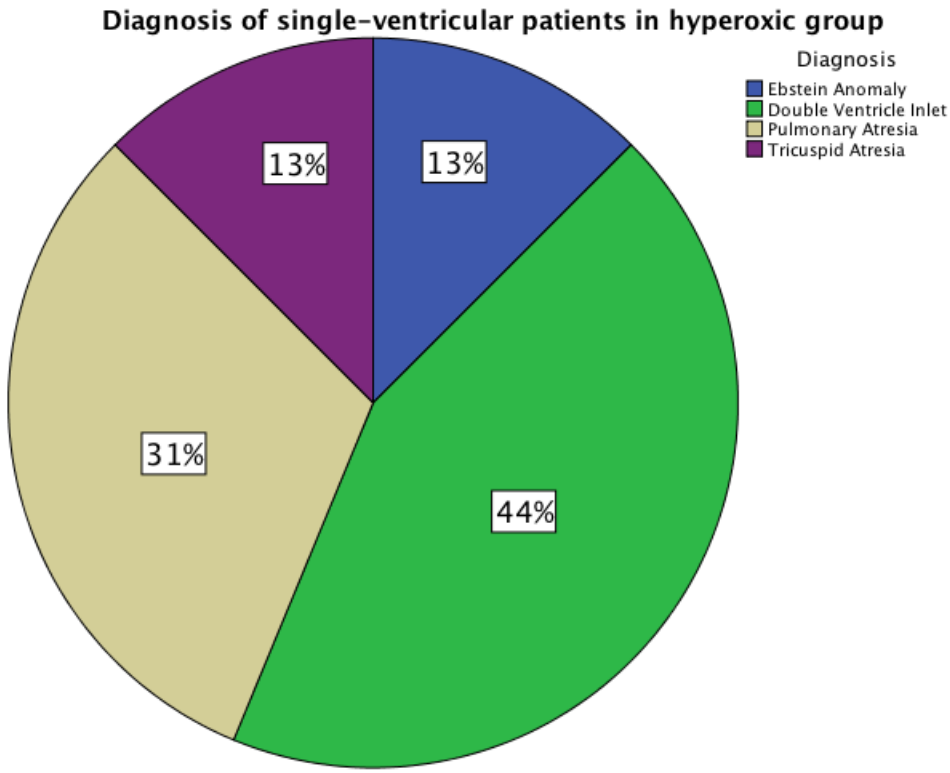
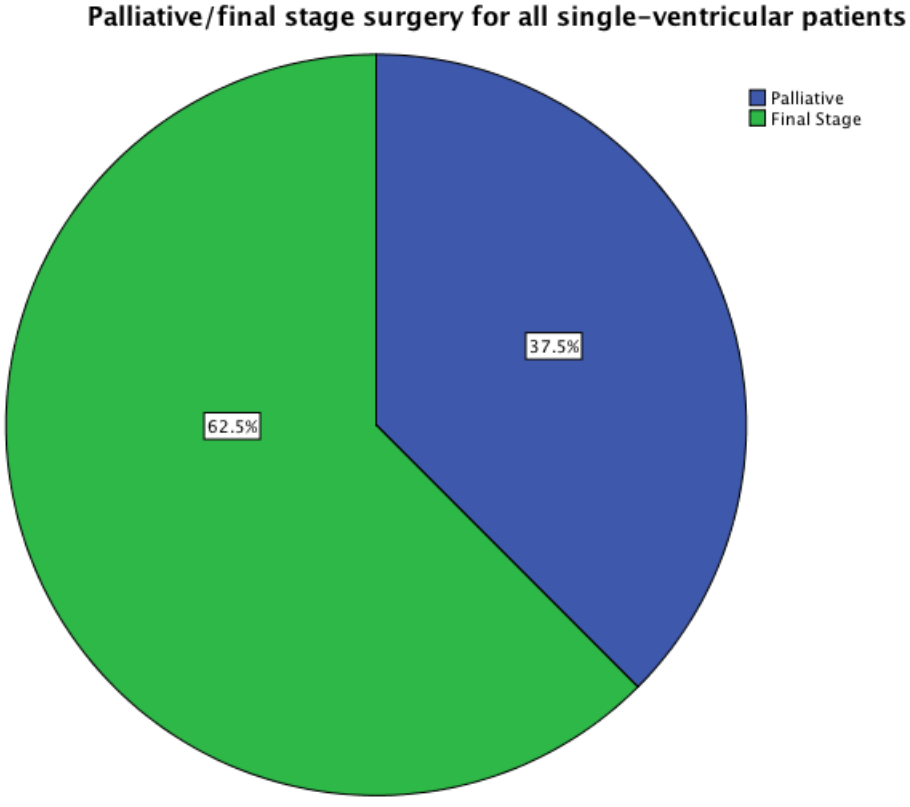
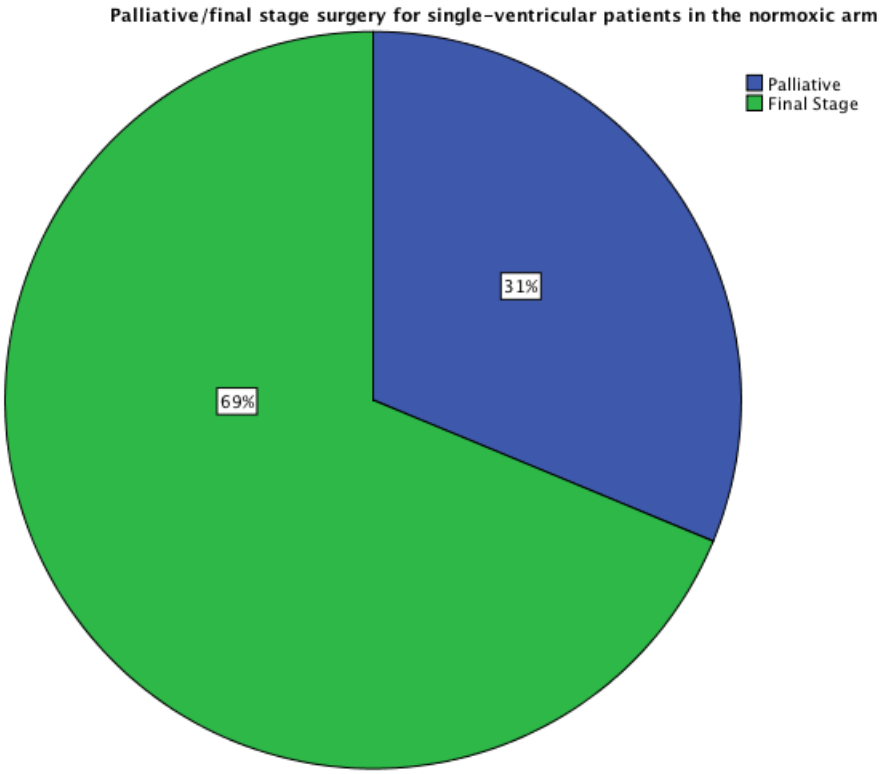


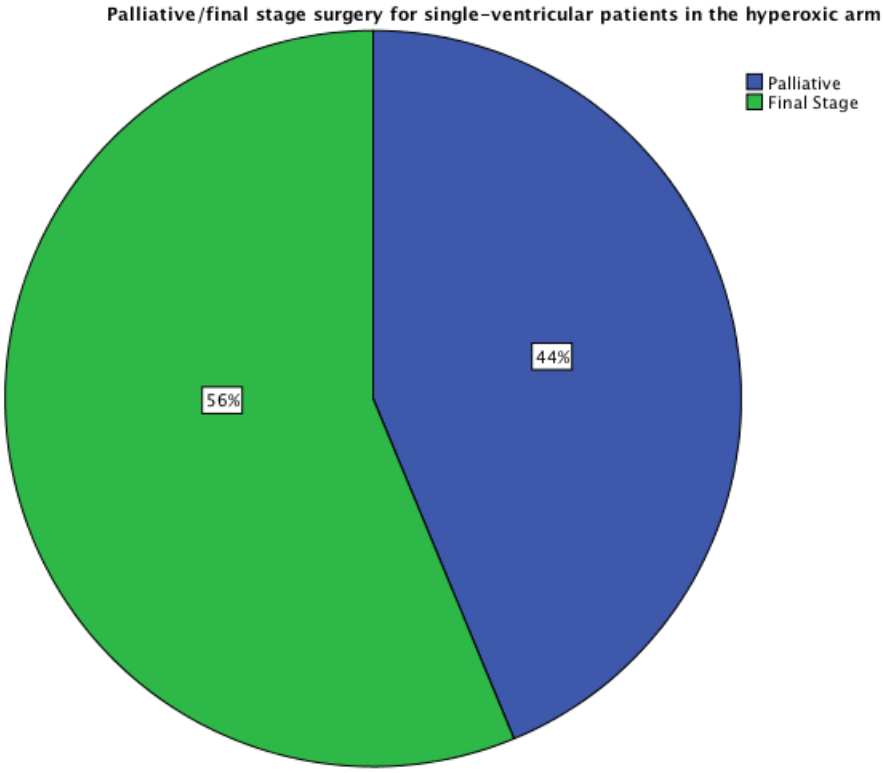
NB. Percentages are rounded to the nearest integer



N.B Percentages are rounded to the nearest integer



N.B Percentages are rounded to the nearest integer



N.B Percentages are rounded to the nearest integer

Pre-operative oxygen saturation (single and double ventricular)					
				Statistic	Std. Error
Normoxic	Single Ventricular	Mean		81.06	1.890
		95% Confidence Interval for Mean	Lower Bound	77.03	
			Upper Bound	85.09	
		5% Trimmed Mean		81.35	
		Median		82.50	
		Variance		57.129	
		Std. Deviation		7.558	
		Minimum		65	
		Maximum		90	
		Range		27	
		Interquartile Range		10	
		Skewness		-.317	.564
		Kurtosis		-.308	1.091
	Double Ventricular	Mean		78.08	2.064
		95% Confidence Interval for Mean	Lower Bound	73.81	
			Upper Bound	82.35	
		5% Trimmed Mean		78.58	
		Median		80.50	
		Variance		102.254	
		Std. Deviation		10.112	
		Minimum		55	
		Maximum		90	
		Range		37	
		Interquartile Range		16	
		Skewness		-.797	.472
		Kurtosis		-.352	.918
Hyperoxic	Single Ventricular	Mean		77.75	1.797
		95% Confidence Interval for Mean	Lower Bound	73.92	
			Upper Bound	81.58	
		5% Trimmed Mean		77.89	
		Median		80.00	
		Variance		51.667	
		Std. Deviation		7.188	
		Minimum		65	
		Maximum		88	
		Range		23	
		Interquartile Range		15	
		Skewness		-.394	.564
		Kurtosis		-1.211	1.091
	Double Ventricular	Mean		81.48	1.116
		95% Confidence Interval for Mean	Lower Bound	79.16	
			Upper Bound	83.79	
		5% Trimmed Mean		81.59	
		Median		82.00	
		Variance		28.625	
		Std. Deviation		5.350	
		Minimum		70	
		Maximum		91	
		Range		21	
		Interquartile Range		7	
		Skewness		-.416	.481
		Kurtosis		-.223	.935

Oxygen Pressure Changes During Cardiopulmonary Bypass for All Patients (Temperature Corrected)

Intervals	Mean pO_2 normoxic	Mean pO_2 Hyperoxic	p value
pO_2 at start of CPB	53.21	166.34	>0.001
pO_2 5 min post CPB	65.13	202.47	>0.001
pO_2 10 min post CPB	73.97	197.17	>0.001
pO_2 30 min post CPB	115.55	172	>0.001
pO_2 immediately post CPB off	161.65	172.21	0.37

Haematocrit Changes During Cardiopulmonary Bypass in All Patients

Intervals	Haematocrit		p value
	Normoxic	Hyperoxic	
Hct at Induction	44.16	45.38	0.50
Hct 10 min post CPB	28.52	28.43	0.94
Hct 30 min post CPB	28.77	28.31	0.71

Oxygen Partial Pressure During Cardiopulmonary Bypass in Single-Ventricular Patients (Temperature Corrected)

Pathology	Intervals	Mean pO_2 normoxic	Mean pO_2 Hyperoxic	p value
Single Ventricle	pO_2 at start of CPB	55.4	167.87	>0.001
	pO_2 5 min post CPB	63.73	169.6	>0.001
	pO_2 10 min post CPB	65.87	171.8	>0.001
	pO_2 30 min post CPB	114.4	155.67	0.001
	pO_2 immediately post CPB off	159.4	173.21	0.461

Haematocrit Changes During Cardiopulmonary Bypass in Single-Ventricular Patients

Pathology	Intervals	Heamtocrit		p value
		Normoxic	Hyperoxic	
Single -Ventricle	Hct at Induction	47.42	49.20	0.47
	Hct 10 min post CPB	31.44	33.02	0.35
	Hct 30 min post CPB	31.39	32.57	0.53

Oxygen Partial Pressure During Cardiopulmonary Bypass in Double Ventricular Patients (Temprature Corrected)

Pathology	Intervals	Mean pO_2 normoxic	Mean pO_2 Hyperoxic	p value
Double -Ventricle	pO_2 at start of CPB	51.78	165	>0.001
	pO_2 5 min post CPB	66	231.47	>0.001
	pO_2 10 min post CPB	79.04	216.2	>0.001
	pO_2 30 min post CPB	116.3	184.25	>0.001
	pO_2 immediately post CPB off	163.18	171.47	0.6

Heamatocrit Changes During Cardiopulmonary Bypass in Double Ventricular Patients

Pathology	Intervals	Heamtocrit		p value
		Normoxic	Hyperoxic	
Double -Ventricle	Hct at Induction	41.90	42.73	0.72
	Hct 10 min post CPB	26.49	25.24	0.25
	Hct 30 min post CPB	26.87	25.36	0.18